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Experiments to Ascertain the Existence of Biochemical Antagonism between L-Ascorbic Acid and Structurally Related Compounds

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Received April 12, 1948

INTRODUCTION

Woolley and Krampitz (1) have reported a condition in mice and cotton rats which shows gross similarity to scurvy. The condition is produced as a result of the administration of highly purified diets containing relatively massive amounts of D-glucoscorbic acid (I), an analog of L-ascorbic acid (II). Using mice, supplementation of the glucoscorbic acid diet with moderate amounts of L-ascorbic acid did not prevent the condition, nor could L-ascorbic acid cure it.

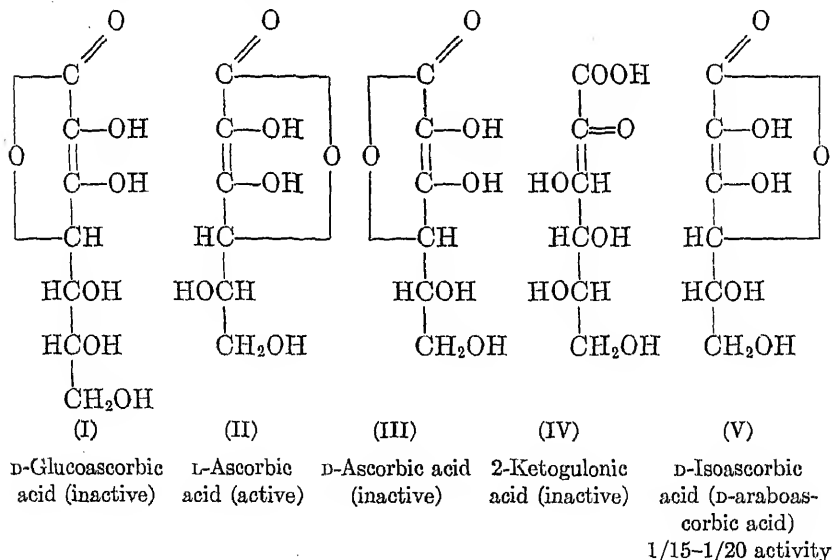
Subsequently, Woolley (2) fed a purified diet, composed of sucrose, casein, salts, vitamins, liver concentrate, and cellulose, to which was added 5-7% of glucoscorbic acid, to guinea pigs which, unlike mice and rats, require an external source of ascorbic acid, and produced a condition characterized by diarrhea, hemorrhage and death. It is claimed that this condition could be prevented by small amounts of L-ascorbic acid. On this basis the condition has been regarded as an ascorbic acid deficiency and the action of glucoscorbic acid as another example of analog antagonism. Banerjee and Elvehjem (3), who reported on similar experiments with rats, chicks, and guinea pigs, found that feeding guinea pigs a diet containing 10% glucoscorbic acid produced a condition characterized by diarrhea but no hemorrhages, and that the condition could not be prevented by simultaneously feeding 10% L-ascorbic acid. They also report that solubilized liver, containing no ascorbic acid, could prevent the condition and suggest that the results observed are due to some factor in liver other than ascorbic acid.

The condition has been described as similar to scurvy on rather equivocal, non-specific bases. No histological or enzymatic data have been presented to characterize the condition.

The present investigation was undertaken to determine, if possible, whether the action of glucoscorbic acid is an example of true metabo-

lite antagonism and whether other analogs of ascorbic acid having no activity¹ or partial activity may compete metabolically with L-ascorbic acid.

In previous investigations (4, 5, 6), it has been established that, for normal osteoblastic function in the guinea pig, a critical ascorbic acid intake is necessary. The "alkaline" phosphatase level of the blood has been shown to be an index of this function. The fall in blood phosphatase level appears to be the most sensitive and earliest specific manifestation of the scorbutic process. Working in the range of this critical level and supplementing the diet with D-glucoscorbic acid, D-ascorbic acid (III), and 2-ketogulonic (IV) acid, all of which are inactive; as well as with D-isoascorbic acid (V) which shows from 1/15th to 1/20th of the activity of L-ascorbic acid (6), an attempt has been made to demonstrate analog antagonism by studying serum phosphatase levels and responses.



Under the conditions of these experiments no antagonisms could be demonstrated between the L-ascorbic acid and any of the inactive compounds. When combinations of subcritical levels of L-ascorbic acid and D-isoascorbic acid, which together constituted a calculated ade-

¹ Activity refers to the ability to prevent the nutritional symptoms or biochemical lesions of scurvy, L-ascorbic acid being taken as 100% activity.

quate dose, were administered, an additive effect was observed, even though relatively large amounts of the weakly active compound had to be given.

Histological examinations of the tooth structure of animals fed large amounts of glucoascorbic acid alone, supplemented, or followed by, minimal amounts of ascorbic acid, also indicate no antagonism.

EXPERIMENTAL METHODS

The methods are similar to those described in previous studies (4-6). Groups of guinea pigs 6-7 weeks of age weighing from 250 to 275 g. were separated into groups of 4 or 5 and fed *ad lib.* a scorbutogenic diet consisting of equal parts of skim milk (heated at 100°C. for 4-5 hrs.), rolled oats and bran. The diet was supplemented by 1 ml. of cod liver oil every 4-5 days. Measured amounts of crystalline L-ascorbic acid and/or the desired analog, dissolved in water immediately before use, were administered by mouth, when desired, by means of accurately calibrated syringes. The animals on the scorbutogenic diet invariably showed evidence of scurvy in 18-25 days, and characteristically low serum phosphatase levels in 18 days. Control animals responded characteristically to the critical level of 0.225 mg. ascorbic acid daily after 5 days administration and, at levels below this, there was no phosphatase response.

Estimation of Serum "Alkaline" Phosphatase

The animals were bled from the heart under very light ether anesthesia. 0.5-1.0 ml. of blood was allowed to clot and the serum collected. Phosphatase estimations were carried out by the micro method previously described (6), employing the method of Shinowara *et al.* (7) for the estimation of inorganic phosphate.

Initial values were taken when the animals were placed on the scorbutogenic diet and found to range between 15 and 35 units. After 18 days on the scorbutogenic diet the base value was determined and selected animals with low values and in good condition were used. The desired compound or mixture of compounds were fed daily, and on the fifth day the final phosphatase determination was made.

RESULTS

D-Glucoascorbic Acid

The results of several typical experiments are indicated graphically. From Fig. 1 it can be seen that, even after maintaining the animals on 100 mg. of glucoascorbic acid daily during the preparative period and feeding 100 mg. of glucoascorbic acid in addition to 0.25 mg. ascorbic acid (just above the critical dose) during the curative period, no antagonistic or competitive effect is apparent under the conditions of this experiment. In this experiment the intake of D-glucoascorbic acid is only about 1% of the diet (100 mg./day) and a natural diet was fed

rather than a synthetic one as in the experiments below. However, the phosphatase response is so sensitive it was felt desirable to make these modifications in this experiment.

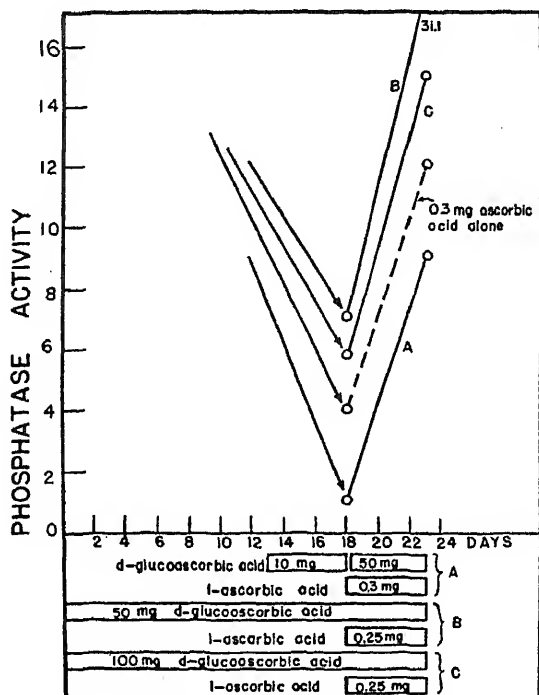


FIG. 1. The influence on the serum phosphatase level of feeding critical doses of L-ascorbic acid to guinea pigs maintained on a scorbutogenic diet supplemented by D-glucoscorbic acid, fed daily in the amounts indicated. The dotted curve indicates the response of scorbutic guinea pigs to 0.3 mg. ascorbic acid daily for 5 days in a control group of animals not fed D-glucoscorbic acid.

Examination of Animals Fed Larger Amounts of Glucoascorbic Acid²

Groups of animals were placed on the diet used by Woolley (personal communication) consisting of: casein 30 parts, cellulose 20 parts, salts 5 parts (8), Wilson's liver fraction L³ 5 parts, glucose 39 parts, and corn oil fortified with vitamins A, D, E, and K (9) and water-soluble vitamins as described by Woolley and Krampitz (1);

² We are grateful to the Wallerstein Laboratories for a liberal supplement to our glucoscorbic acid supplies.

³ We are grateful to the Wilson Laboratories for a liberal supply of liver fraction L.

D-glucoscorbic acid plus one equivalent of sodium bicarbonate was added, to the extent of 5% by weight, at the expense of the glucose. The animals were weighed and examined daily. Their food consumption was determined and indicated an average D-glucoscorbic acid intake of about 660 mg. daily. At the termination of the experiment, the jaw bones were dissected out and the histological tooth structure was studied.⁴ Animals were selected from groups fed (1) the diet alone; (2) the diet supplemented by 0.3 mg. ascorbic acid daily for 18 days, after which the ascorbic was withdrawn; (3) the diet without glucoscorbic acid; and (4) the diet with glucoscorbic acid followed by 4 doses of 0.3 mg. of ascorbic acid. The dosage of 0.3 mg. ascorbic acid daily was selected since this produces a mild degree of healing in a scorbutic animal and only partially protects a guinea pig against scurvy. It is, however, adequate to produce a phosphatase response. Antagonism, if it occurs, ought to be easily apparent at this critical level.

The examinations indicated that the response that results from the administration of the minimal dose of 0.3 mg. per day for 4 days is comparable to that resulting from similar supplements added to the usual scorbutogenic diet without glucoscorbic acid. Feeding minimal amounts of ascorbic acid (0.3 mg. daily) simultaneously with 5% glucoscorbic acid results in the very mild histopathology usually associated with this subminimal protective dose, 0.5 mg. daily being required for protection. The withdrawal of the small daily supplement resulted in pronounced scorbutic changes typical of frank scurvy.

It may be concluded that large amounts of D-glucoscorbic acid fed simultaneously with minimal amounts of L-ascorbic acid exert no apparent antagonistic action to the ascorbic acid, nor is prior saturation of the animal with D-glucoscorbic acid effective in antagonizing the curative effect of small amounts of ascorbic acid as evidenced by histological examination. This however does not exclude the possibility that D-glucoscorbic acid antagonizes L-ascorbic acid in certain biological systems, or that certain of the scurvy-like signs may be the result of such antagonisms.

D-Isoascorbic Acid

It has been suggested by Zilva (10) that the inactivity of certain of the analogs of ascorbic acid is due to the inability of the tissues to fix the compounds, and they are rapidly excreted. Since D-isoascorbic acid does have some activity, it seemed a desirable compound to administer

⁴ The histological examination was made and interpreted by Dr. Henry M. Goldman, oral pathologist, Beth Israel Hospital, Boston, Mass., to whom we express our sincere thanks.

alone as well as along with L-ascorbic acid (6), and, since subcritical doses are ineffective in altering or maintaining the phosphatase level, about 4.5 mg. of the D-isoascorbic acid are necessary for protection. Two mg. are equivalent to about 0.1 mg. ascorbic acid and could safely be used as a preparative dose, which then could be supplemented with varying amounts of L-ascorbic acid. The preparative doses were given to saturate the tissues as much as possible before administering the

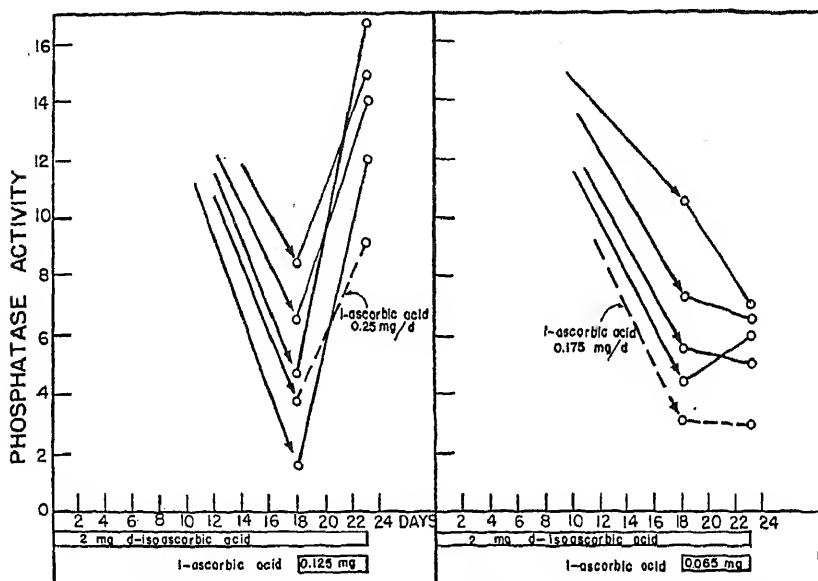


FIG. 2. The influence on the serum phosphatase level of guinea pigs of feeding subcritical doses of D-isoascorbic acid (1/20 activity of L-ascorbic acid), then supplemented by small doses of ascorbic acid which, together with the D-isoascorbic acid, constitutes the equivalent of just over a critical dose (0.225 mg.), or just under this dose. The dotted curves indicate the decline of the phosphatase level in a control group of animals receiving 0.175 mg. ascorbic acid daily and the rise when 0.25 mg. is administered daily.

L-ascorbic acid. The results indicate that, in spite of the relatively large amount, in total weight, of D-isoascorbic acid administered along with the small amounts of L-ascorbic acid, there is no antagonism, and that (Fig. 2) subcritical levels of each when given together result in an elevation of the phosphatase level similar to that which would be expected from the equivalent in terms of L-ascorbic acid.

D-Ascorbic Acid

The ineffectiveness of *D*-ascorbic acid as an antiscorbutic agent has long been known (11). As a result of the unavailability of the compound at the time that an earlier general study (6) was being made of the phosphatase response to many analogs of ascorbic acid, it could not be tested. Professor W. N. Haworth has generously supplied us with a small sample for which we extend our thanks. A study of the compound according to the methods previously used (6) indicates that it has no influence in raising the serum phosphatase level of scorbutic guinea pigs, emphasizing the specificity of the phosphatase response for anti-scorbutic compounds (Fig. 3b).

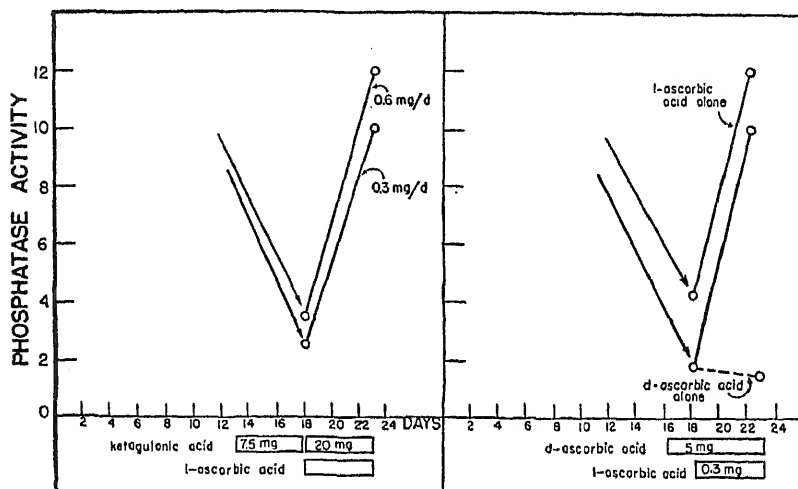


FIG. 3a. The influence of administering 2-ketogulonic acid on the serum phosphatase level of scorbutic guinea pigs which are fed small doses of L-ascorbic acid.

FIG. 3b. The influence of feeding *D*-ascorbic acid on the serum phosphatase response of scorbutic guinea pigs to critical doses of *L*-ascorbic acid. The broken curve indicates the ineffectiveness of *D*-ascorbic acid alone.

Due to the small amount of material available, an experiment using 2 animals was carried out to determine any possible antagonism between the *D*- and *L*-forms of ascorbic acid. The animals were given two preparative doses of 5 mg. of *D*-ascorbic acid daily followed by 5 doses to which was added 0.3 mg. of *L*-ascorbic acid. The response as illustrated in Fig. 3b indicates no apparent antagonistic effect under the conditions of the experiment.

2-Ketogulonic Acid

The compound, which is an intermediate in the technical synthesis of L-ascorbic acid and is the stable reductant of 2,3-diketogulonic acid, which results from the opening of the lactone ring of L-ascorbic acid, has been found to be inactive (6). Its close relationship to L-ascorbic acid suggested possible antagonism. The results of an experiment (Fig. 3a) indicate no such antagonism.

CONCLUSIONS

The results of the experiments described indicate that there is no apparent antagonism between L-ascorbic acid and D-glucoascorbic acid, even when the latter is fed in massive amounts, nor is there apparent antagonism between L-ascorbic acid and 2-ketogulonic acid, or with relatively large amounts of the weakly active D-isoascorbic acid. Limited experiments with D-ascorbic acid confirm the specificity of the phosphatase response for antiscorbutic substances and indicates no apparent antagonism between inactive D-ascorbic acid and active L-ascorbic acid. The results are based on the serum phosphatase response to guinea pigs to critical quantities of antiscorbutic substances and on confirmatory histological examinations.

The condition resulting from the massive feeding of D-glucoascorbic acid appears not to be an example of metabolite (ascorbic acid) antagonism as has been generally suggested. This conclusion based on histological examination does not exclude the possibility that D-glucoascorbic acid does antagonize L-ascorbic acid in certain biological systems.

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Reaction of Borate with Polysaccharides: Blood Group Substance from Intestinal Mucosa and Gastric Mucin

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INTRODUCTION

The separation of a polysaccharide fraction from a calf intestinal mucosa preparation by borate precipitation has been reported (1, 2), and the polysaccharide subsequently purified and shown to be related to the blood group substances (3). The blood group substance in commercial hog gastric mucin was also amenable to borate precipitation. The application of the borate procedure, the implications of the method, and some of the properties of the products are reported herein in greater detail.

EXPERIMENTAL

Polysaccharide from Intestinal Mucosa

Calf intestinal mucosa was treated by a procedure for obtaining phosphoesterase (4), the essential steps being digestion with trypsin in the presence of toluene, and successive precipitations with ammonium sulfate and with acetone. This phosphoesterase preparation, some of which was generously supplied by the Armour Laboratories, was fractionated with borate as illustrated by the following example. Four-tenths g. of the phosphoesterase preparation was dissolved in 15 cc. of water; 20 cc. of saturated ammonium sulfate were added and the mixture left at room temperature for several hours. The precipitate, inappreciable in some instances, was removed by centrifugation or filtration. Seven volumes of the cleared solution were mixed with one volume of 0.2 *M* sodium tetraborate (kept at 37°C. for complete solution). The mixture was kept at 7°C. for one hour and stirred vigorously to convert the gel which first formed to a flocculent precipitate. The precipitate forms slowly, and quantitative precipitation was usually shown by a clear supernatant fluid. The greater part of the phosphoesterase was in the solution. The precipitate was removed by centrifuging, dissolved in about 30 cc. of water and dialyzed at 7°C. against two successive 2-l. portions of water to eliminate the ammonium sulfate and borate. The dialyzed solution was mixed with 2 volumes of acetone and sufficient 2 *M* sodium acetate (about 0.4 cc.) was added to produce a flocculent precipitate. The precipitate was collected by centrifug-

ing, washed once with acetone, and dried in a stream of air. The yield varies, with different starting preparations, from 20 to 40%. In the experiment just described, the solution was 0.5 saturated with ammonium sulfate. Other experiments have shown that as little as $\frac{1}{3}$ saturation with ammonium sulfate is sufficient to precipitate the polysaccharide when borate is present. These preparations, although referred to as polysaccharides, are probably polysaccharide-protein compounds as appears to be the case with the blood Group A substance.

The above precipitation with borate was repeated, the phosphoesterase content being reduced to one unit (4) per 10 mg. or less. Treatment with $C\gamma$ alumina (4) removed most of the color which contaminated some preparations. Further purification was accomplished by deproteinization with chloroform-amyl alcohol (5): a 1.5% solution of the polysaccharide in 0.07 M $NaHCO_3$ was mixed with 0.25 volumes of chloroform and 0.1 volume of amyl alcohol, and stirred vigorously in a closed vessel for 15 mins. This treatment was repeated on the aqueous supernatant fluid, which was separated by centrifugation. Five treatments were required for deproteinization of the preparation described. The polysaccharide was, after dialysis, precipitated by the addition of 2 volumes of acetone and 1.0 cc. or more of 2 M sodium acetate/20 cc. of aqueous solution. The precipitate was distinctive in character, forming slowly in dense sticky masses on the sides of the vessel and on the stirring rod. It was readily dried by triturating in acetone with a spatula. The product was white and granular.

From the intestinal mucosa of the hog (starting materials supplied by the VioBin Corporation) and the dog, phosphoesterase preparations were also obtained. From these materials, polysaccharide preparations were obtained by precipitation with borate, only the initial precipitation being performed. The yields were 16 and 25%, respectively.

Polysaccharide from Hog Gastric Mucin

Because of the suspected relationship of the borate-precipitating mucosa polysaccharides to the blood group substances, the borate fractionation procedure was applied to commercial hog gastric mucin (Wilson Labs. Type 1701-W. Lot 52758), a recognized source of blood Group A substance (6, 7). In 173 cc. of water and 27 cc. of 0.5 M $NaHCO_3$, were suspended 2.0 g. of mucin. As preservative, 1.0 cc. of chloroform was added and the mixture was left for 18 hrs. at room temperature and stirred occasionally. The mucin was almost completely dissolved, and only a small amount of sediment was removed from the milky solution by centrifuging. 124 g. of ammonium sulfate, the amount necessary to precipitate the proteins, were added to this solution; the large amount of precipitate which formed was removed by filtration. Seven volumes of the clear solution were mixed with one volume of 0.2 M sodium tetraborate, left at 7°C. for 2-3 hrs. and stirred occasionally. The precipitate was collected by centrifuging, dissolved in 30 cc. of water, dialyzed, and reprecipitated with acetone and acetate. The character of this precipitate resembled that of the purified calf mucosa polysaccharide. The yield was 11.5%. This preparation was free of protein without further treatment, as shown by the chloroform-amyl alcohol test.

Electrophoretic Properties of the Polysaccharides

For the determination of the electrophoretic properties of the polysaccharides 2.0% solutions in phosphate buffer solution of pH 7.6,

ionic strength 0.2, containing 0.15 *M* NaCl were used. A potential gradient of 5.3 volts cm.⁻¹ was used in all experiments. Migration was anodic in every instance. The calf mucosa polysaccharide (Preparation I) gave evidence of only one component throughout 5 hrs. of electrophoresis, as is shown by the concentration gradient curve in Fig. 1, upper curve, taken at 4 hrs. The mobility, calculated from the descending arm, was 3.3×10^{-5} cm.² sec.⁻¹ volt⁻¹. In additional experiments, in which 1% solutions of the polysaccharide were used, the mobility was found to be constant from pH 8.5 to pH 4.7; at pH 3.0, in 0.15 *M* NaCl buffered with 0.027 *M* sodium phosphate-sodium citrate, it was 2.7×10^{-5} . The hog mucin polysaccharide, in the same concentration and under the same conditions as the initial experiments with the

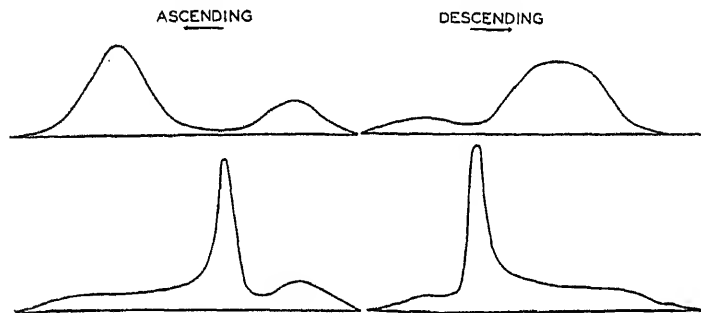


FIG. 1. Concentration gradient curves of polysaccharides at pH 7.6 after migration for 4 hours. Upper curve: calf mucosa polysaccharide; lower curve: hog mucin polysaccharide (blood Group A substance).

calf mucosa polysaccharide, showed two components. The concentration gradient curves at the end of 4 hours are shown in Fig. 1, lower curve. The mobility of the faster component was 3.9×10^{-5} cm.² volt⁻¹ sec.⁻¹; that of the slower component was 1.5×10^{-5} . The slower component, constituting 65% of the preparation, had the same mobility as was found by Bendich *et al.* (8) for blood Group A substance from the same source. The specific optical rotations of essentially homogeneous preparations of blood group substance from hog gastric mucin have been reported to be dextrorotatory (7, 9), whereas ours had a rotation of -20° . This suggests that the faster component might be the "acid" polysaccharide isolated from the same source by Meyer *et al.* (10), which had a specific rotation of -35.7° .

Chemical and Serological Properties of the Polysaccharides

Some of the properties of the polysaccharides isolated by borate precipitation are given in Table I. The precipitation with Type 14 serum is expressed as the maximum dilution of polysaccharide giving a precipitate with Type 14 horse antipneumococcus serum (supplied

TABLE I

Chemical and Serological Properties of Polysaccharides Prepared by Borate Precipitation and of other Preparations of Blood Group Substance

Preparation	Volatile matter ^b	Nitrogen	Reducing sugar ^c	Ash ^d	[α] _D ²⁰	Precipitation with Type 14 serum	Inhibition of isoagglutination	
							Type A	Type B
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>					
Calf intestinal mucosa, purified I	7.3	5.33	51.0		+9°	1:10,000	0.02	0.2
Calf intestinal mucosa, purified II	4.8	5.59	53.9	1.16			0.02	0.1
Hog gastric mucin, purified	10.0	4.65	58.6	1.34	-20°	1:50,000	0.02	50
Hog gastric mucin, original							0.10	150
Hog intestinal mucosa, unpurified						1:10,000		
Dog intestinal mucosa, unpurified						1:1,000		
Blood Group A and B substance ^a						1:50,000	0.05	0.05

^a A mixture of A and B substances supplied by Sharp and Dohme.

^b All analytical values have been corrected for the volatile matter.

^c The reducing value, as glucose, was determined colorimetrically with the new copper reagent of Somogyi (12). The samples, after solution in 5.0 cc. of water and 0.5 cc. of concentrated HCl, were hydrolyzed by heating for 2 hrs. at 100°C. in closed tubes.

^d Ashed with sulfuric acid; ash calculated as sodium equivalent.

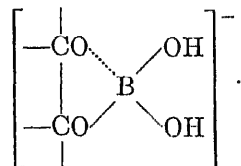
by the New York City Department of Health, Bureau of Laboratories). Two-tenths cc. portions of various dilutions of the polysaccharides in 0.9% NaCl were mixed with equal portions of serum; the mixtures were incubated at 37°C. for one hour, and left at 7°C. for 18 hrs. The

amounts of precipitate were estimated after centrifuging the tubes in the cold. None of the materials tested gave any precipitate with normal horse serum. Type 14 pneumococcus antiserum (horse) was used as a reagent to compare the polysaccharides studied, as the relationship of the Type 14 polysaccharide and the blood group polysaccharides is well established (11). The striking effect of temperature shown by precipitation of the blood group polysaccharide and Type 14 serum (11, 6) was also shown by the polysaccharide from calf intestinal mucosa.

The inhibition of isoagglutination is expressed as the micrograms of polysaccharide which inhibited agglutination in the following system: 0.1 cc. of each polysaccharide dilution was added to 0.1 cc. of diluted serum. The dilutions of serum were chosen so that 0.1 cc. contained twice the amount of agglutinin necessary to agglutinate the red cells used. This was a dilution of 1:20 for the agglutination of Type A cells, and 1:5 for the agglutination of Type B cells. The mixture of polysaccharide and diluted serum was allowed to stand at room temperature for 15 mins. One-tenth cc. of a 0.5% suspension of red cells was then added to each tube. The tubes were allowed to stand for one hour at room temperature, centrifuged lightly, the contents gently resuspended, and a portion examined microscopically. This is essentially the procedure followed by Morgan and King (7).

DISCUSSION

The interaction of borate and polysaccharide probably represents an extension of the well-known (13-19) interaction of borate and simple polyhydroxy compounds which takes place with an enhanced ionization of the borate. It is generally considered that boric acid, which, in solution, will be in equilibrium with tetraborate, reacts with two adjacent hydroxyl groups on the same side of a carbon chain to give the structure



The remaining hydroxyl groups on the boron can, in turn, coordinate with another pair of organic hydroxyl groups. If the reaction with polysaccharide is similar, then some of the component carbohydrates of the

polysaccharide must contain adjacent hydroxyl groups in the *cis* position. Further, because of the ability of borate to unite with two molecules of carbohydrate, chain-like structures are a possibility with polysaccharides because of the multiple reacting groups which they contain. The hypothesis of a polysaccharide-borate complex is supported by the finding that the polysaccharide in a borate solution has an enhanced electrophoretic mobility and the solution is more viscous (2). Morgan and King (7) have observed that blood Group A substance from hog gastric mucin becomes more viscous with borate. The enhanced viscosity in both instances probably represents the formation of chain-like structures. The decreased solubility of the polysaccharide in ammonium sulfate when borate is present is consistent with a picture of complex formation, probably resulting from the masking of the hydrophilic hydroxyl groups by the borate radical. Complex formation is readily reversed, for the borate can be removed by dialysis against water.

The substances investigated in the present study are probably polysaccharide-protein compounds but interaction of borate with protein-free polysaccharides occurs as well (20), although the effect on solubility has not been investigated. A reaction between borate and the polysaccharide hyaluronic acid from bovine vitreous humor could not be shown by the methods used in the present study. This may concern spatial structure, for hyaluronic acid is composed of N-acetyl-D-glucosamine and D-glucuronic acid, neither of which contains adjacent hydroxyl groups in the *cis* position. Blood group substance from hog gastric mucin, on the other hand, contains D-galactose and L-fucose (8), both of which meet this requirement. A recent paper (20) describes the reaction of boric acid with polysaccharides, principally vegetable gums, as measured by gel formation. In every case, only those polysaccharides reacted which contained carbohydrates that met the above structural requirement.

The inhibition of blood group isoagglutination by the calf intestinal mucosa polysaccharide further serves to identify this material as blood group substance or substances, closely related to both Type A and Type B, the Type A activity being 10 times greater. The polysaccharide isolated from the fourth stomach of cattle by Jorpes and Thaning (21) was 100 times more active in neutralization of Type A agglutination than of Type B agglutination. The serological data in Table I show that the preparation obtained from hog gastric mucin by the borate

procedure is specific blood group substance, strongly inhibitory of Type A agglutinins, although some reaction with Type B agglutinins occurs. Preparations from this source contain O substances also (8). The 5-fold increase in the ability of the hog mucin preparations by the borate method to neutralize Type A agglutinins in a fraction representing only 11.5% of the starting material, indicates that a satisfactory concentration of blood group substance has occurred.

The isolation of blood group substances from the intestinal mucosa is not surprising, for it has already been shown that the small intestine of man is rich in these substances (22). The relationship of the blood group substances of different species (hog, horse, man) has been reported (23, 21). Recent studies (21) show that cattle also contain related blood group substance; preparations had been obtained from the fourth stomach (abomasum) of cattle which contained both a neutral and an acidic (ester of H_2SO_4) polysaccharide, each of which inhibited blood group agglutinins. The present studies show that the small intestine of cattle is another source, but in this case apparently only the H_2SO_4 ester is obtained as indicated by the negative ionization at low pH values.

SUMMARY

A polysaccharide related to the specific blood group substances was isolated from a calf intestinal mucosa phosphoesterase preparation by precipitation with borate from a solution containing ammonium sulfate. The borate precipitation method was also used to obtain blood group substance from commercial hog gastric mucin. The electrophoretic, chemical, and serological properties of the polysaccharides, as well as of the cruder preparations from hog and dog intestinal mucosa, were investigated. The interaction of borate and polysaccharide is believed to be an extension of the well-known interaction of borate and simple carbohydrates.

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Some Quantitative Analyses of the Particulate Fractions from Mouse Liver Cell Cytoplasm ¹

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INTRODUCTION

The demonstration by Bensley and Hoerr (1) that mitochondria could be prepared from a cytoplasmic extract of guinea pig liver by centrifugal methods, and the extensive work of Claude (2-6) on the fractionation of cytoplasmic particulates from various tissues by differential centrifugation have made possible the study of cellular particulates, even though these particulates could not be visualized in the light microscope. The procedure presumably allows for the isolation of these particulates in a state closely approximating that in which they existed within the cell.

This investigation was undertaken to study the particulates as components of cells, and thus was primarily directed at yields and quantitative interrelationships, and secondarily at purity.

MATERIALS AND METHODS

Animals

Normal mice, fasted 18-24 hrs. (2), generally of the A or C3H strains or their hybrids, between one and two months of age were used.²

Preparation of Centrifugal Fractions

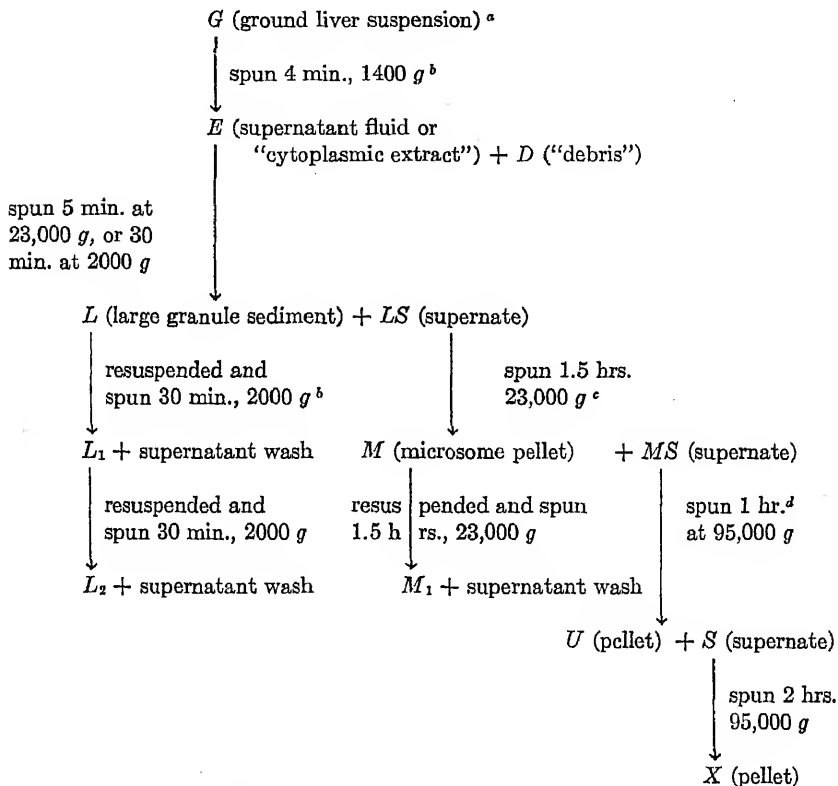
Livers were removed under ether anesthesia after clamping the portal vein to allow for drainage of blood. They were harvested into an iced beaker, forced through a cold

¹ Aided by grants from the University of Minnesota Graduate School, the Donner Foundation, and the American Cancer Society.

² We are indebted to Dr. J. J. Bittner for supplying the animals used in this study.

tissue press with holes about 1 mm. in diameter, weighed, and transferred to a cold mortar in an ice-water bath. Grinding and extraction was carried out essentially according to Claude (2).

The differential centrifugation procedure employed (essentially following Claude (2)) is schematically presented in Fig. 1. Claude's terminology is, in general, adhered to, although this does not imply that specific fractions are as homogeneous as the names imply.



^a Each g. wet tissue ground with 4 cc. alkalized saline (2).

^b Servall angle centrifuge operated in refrigerator. All gravitational fields calculated for center of tubes.

^c Multispeed attachment to International centrifuge operated in cold room at -15°C .

^d Air driven ultracentrifuge (7), operated in a vacuum. Head chilled to 0°C . before run.

FIG. 1. Fractionation of ground liver suspension by differential centrifugation.

In carrying out the washing and purification of a particulate fraction, we have not felt it necessary or advisable to employ a short clearing run after resuspension of the fraction, nor have we discarded residues of sediments (2) since we wished as complete recovery as possible. Observations in the electron microscope have failed to show any significant morphological differences between the material sedimented in the clearing run and that subsequently sedimented under the original conditions, except in the degree of aggregation of the individual particles. It has proved very difficult to resuspend a fraction, such as microsomes, in saline, without leaving a large fraction, frequently half or more, in an aggregated state. On the other hand, if microsomes are resuspended in distilled water and then cleared for 5 mins. at 23,000 *g*, only a very small fraction is sedimented.

Analytical Procedures

Phosphorus was determined according to the method of Fiske and SubbaRow (8), and nitrogen according to the Pregl micro Kjeldahl method (9). Total lipide was determined by extracting aliquots of the various fractions with 25 volumes of boiling alcohol-ether (3:1) and then re-extracting with petroleum ether, drying, and weighing. Ribose was estimated by the orcinol-HCl reaction, essentially as described by Brown (10), in which the primary interfering substance, glucose, is corrected for by determining, at two different wavelengths, the optical density of the color produced.

RESULTS

Effectiveness of Grinding and Extraction Procedures

Table I summarizes the data of a typical experiment concerned with nitrogen distribution and recovery.

TABLE I

Recovery of Cellular Nitrogen in Various Cell Fractions

17.6 g. liver tissue from 26 fasted ZBC mice between 4 and 5 weeks of age treated as indicated.

Fraction	N recovered ^a	Whole cell N recovered	Cytoplasmic N recovered
	<i>mg.</i>	<i>per cent</i>	<i>per cent</i>
<i>G</i>	616	100	—
<i>E</i>	436	70.8	100
<i>W^b</i>	61.8	10.0	—
<i>L₂</i>	71.9	11.7	16.6
<i>M₁</i>	78.3	12.7	18.0
<i>U</i>	15.8	2.6	3.6
<i>S</i>	180	29.2	41.3

^a Actual amount recovered. Losses occur in incomplete decantation of supernates and in subsequent washing of particulates.

^b Supernate obtained by resuspending "debris" and again centrifuging 4 mins. at 1400 *g*.

The cytoplasmic nitrogen found in *E* amounted to 70.8% of the total cell nitrogen, and one wash of the "debris" removed another 10%. This implies that upwards of 86% of the cells were broken by the grinding procedure employed, since liver cell nuclei occupy 6% of the volume (11) and probably account for at least 6% of the nitrogen of the cell.

The 3 particulate fractions actually isolated accounted for 38.2% of the cytoplasmic nitrogen in *E*.

Chemical Composition of Cytoplasmic Fractions

The fractionation procedure was designed to recover as quantitatively as possible the various particulate fractions in the original "cytoplasmic extract." This was done in an attempt to bring out more clearly the contribution of each particulate fraction to the total extract, and thus in Table II are reported the results of various analyses for that amount of each cytoplasmic fraction that could be obtained from 1 g. of liver tissue. This affords a ready means of visualizing, *e.g.*, how much of the cytoplasmic phosphorus is associated with microsomes. In the same table, however, we have described each fraction by defining its per cent nitrogen, per cent phosphorus, *etc.*, so that the characteristic chemistry of a given fraction may be readily compared with that of any other fraction.

The 3 particulate fractions obtained from the cytoplasmic extract are quite distinct from each other. For example, although the *M* and *U* fractions have N:P ratios that are rather similar, these two fractions are quite different when one considers their N:ribose ratios or their ribose:P ratios (ribose-P as % total P).

The fact that these particulate fractions are chemically quite distinct does not necessarily imply that each fraction is homogeneous with respect to particle size or composition. Observation of a microsome fraction in the electron microscope shows 2 morphologically distinct types of particles. Furthermore, if *MS*, the supernate after spinning 90 mins. at 23,000 *g*, is replaced in the multispeed head and spun again for 90 mins. at 23,000 *g*, a pellet is sedimented which accounts for about 30% of the weight of *U*, but has a chemical composition intermediate between that of *M* and *U*. This would indicate that, as far as the submicroscopic particles are concerned, there may be an almost continuous spectrum of particle size or sedimentability, and that the divisions made here are rather arbitrary.

TABLE II
The Chemical Composition of Cytoplasmic Fractions of Normal Mouse Liver

	Mg./g. wet weight of tissue ^c			Rib. P as per cent tot. P	N/P	N/rib.	Composition of fractions based on their dry weight				
	N	P	Ribosed (×2)				N	P	Nucleic acid (based on ribose)	Lipide ^b	Phospho- lipide as per cent tot. lipide ^b
<i>E</i>	27.1 (9) ^a 23.2-30.1	2.64 (6) 2.43-2.92	5.4 (6) 4.5-6.3	42.3 (6) 38.0-45.9	10.3 (6) 9.6-10.5	5.0 (6) 4.7-5.45	0.88 (4) 0.81-0.93	3.9 (4) 3.8-4.2	47.3 (2) 46.9-47.6	15.7 (2) 14.2-17.1	
<i>L</i> ₂	4.83 (13) 3.5-7.2	0.43 (13) 0.32-0.63	0.64 (13) 0.40-0.93	30.6 (13) 24.2-35.8	11.2 (13) 9.6-12.4	7.8 (13) 6.4-11.1	1.11 (6) 0.99-1.19	3.7 (6) 3.2-4.0	27.4 (7) 26.1-28.8	56.6 (7) 51.0-59.5	
<i>M</i> ₁	5.02 (15) 3.9-6.2	0.89 (15) 0.72-1.12	1.93 (15) 1.7-2.2	44.9 (15) 38.3-51.8	5.6 (15) 5.3-6.1	2.6 (15) 2.2-3.1	1.87 (7) 1.6-2.0	9.1 (7) 7.4-10.4	35.1 (7) 33.9-36.5	62.7 (7) 61.0-64.2	
<i>U</i>	1.54 (13) 1.0-3.1	0.25 (13) 0.15-0.51	0.92 (13) 0.48-1.66	78.4 (13) 61-115	6.4 (13) 5.1-8.9	1.7 (13) 1.2-2.3	1.9 (3) 1.4-2.2	16.0 (3) 11.5-19.5	16.8 (5) 10.9-26.6		
<i>S</i>	16.3 (11) 14.0-18.5	0.93 (11) 0.80-1.08	0.80 (9) 0.51-1.15	17.7 (9) 10.6-26.8	17.5 (11) 16.3-19.6	21.8 (9) 14.7-33.8	0.73 (3) 0.71-0.76	1.8 (3) 1.3-2.0			
<i>X</i>	0.86	0.033	0.14	87.7	25.8	6.8					

^a Figures are averages of the number of individual experiments shown in parentheses. Ranges are shown below each average.

^b We wish to thank Dr. Norman Kretchner for these lipid analyses. Phospholipide calculated as lipid P × 25.

^c All calculations based on assumption that supernatant fluid decanted from a pellet contains same concentration of non-sedimented constituents as did original extract.

^d A factor of 2 is used in calculating ribose because only 50% of the ribose of yeast nucleic acid is measured by the orcinol-HCl reaction.

The ratio of N:ribose decreases markedly from *L* to *M* to *U* (Table II). These 3 particulate fractions contain 81% of the ribose of the 4 cytoplasmic fractions, but only 41% of the nitrogen.

The conditions arbitrarily chosen for sedimenting the *U* fraction appear to be rather critical, as indicated by the ribose content of the *X* and *U* fractions. It would appear that a centrifugal field of 95,000 *g* for one hour had effected a maximum concentration of ribose, and that beyond this point more "protein" than "nucleoprotein" was being sedimented. The fact that the conditions chosen for isolation of *U* appear to be at such a critical point may account for the rather large variations encountered in the *U* fraction.

DISCUSSION

Claude (2) has tentatively concluded that the nucleic acid found in a liver extract is associated with the large granules and microsomes of that extract. The analyses given for the ultracentrifugal fraction, *U*, reported in this paper, indicate that a sizeable portion of the cytoplasmic nucleic acid is associated with this particulate fraction. It seems significant to point out again that, while 81% of the cytoplasmic "ribose" is sedimentable, only 41% of the cytoplasmic nitrogen is associated with these particulate fractions.

The most striking differences between the large granule fraction and the smaller particulate fractions, appears to be in the N:P and N:ribose ratios. The large granule fraction appears to contain a good deal more protein per unit of nucleic acid. The two smaller particulate fractions seem to be well differentiated on the basis of their lipid contents—the microsomes containing twice as much lipid as the particles of the *U* fraction. It should be borne in mind, however, that the separation of *M* from *U* is purely arbitrary and that the procedure outlined in this paper is, perhaps, only cutting a continuous spectrum of small particles at some arbitrary point. Claude (2) states that the microsomes from rat liver contain 40–45% lipid, a figure significantly above the 35% that is reported here for mouse liver microsomes. This may mean that Claude is cutting the "continuous spectrum" at a slightly different point than we are, or it may be a reflection of the methods of lipid extraction which he has presumably used. His lipid method, as outlined in an earlier paper (12), involved extraction with acetone and alcohol-ether, and he reported as lipid all the ma-

terial soluble in these solvents. We report as total lipid only that material in an alcohol-ether extract which is subsequently soluble in petroleum ether.

ACKNOWLEDGMENT

We wish to express our sincere appreciation to Miss Florence Holst and Mrs. Ollie Oslon for their invaluable help in carrying out the many analyses reported.

SUMMARY

A detailed description has been presented for the isolation of 3 particulate fractions from the cytoplasm of mouse liver tissue by means of differential centrifugation.

These fractions have been chemically characterized by determinations of their nitrogen, phosphorus, "ribose," and lipid contents.

The large granule fraction, sedimenting in 5 mins. at 23,000 *g*, is rich in phospholipid but poor in nucleic acid. The microsome fraction, which sediments in 90 mins. at 23,000 *g*, contains large amounts of phospholipid, but is also relatively rich in nucleic acid. Finally, the fraction which is sedimented at 95,000 *g* for 1 hr. is relatively poor in lipid but rich in nucleic acid.

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Preparation and Properties of Corn Cob Holocellulose ¹

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INTRODUCTION

Corn cobs represent a continuously replenished reservoir of carbohydrate raw material which might become of increased industrial importance if more information were obtained regarding the various constituent carbohydrates. These carbohydrates, which are almost entirely of polysaccharide nature, constitute about 80% of the cob. The hemicellulose polysaccharides of plants are sometimes isolated from crude plant material by extraction with alkaline solutions. However, lignin interferes, not only because it retards complete solution of the hemicellulose, but also because it dissolves in the extract and thereby causes difficulty in the purification of the hemicellulose. These disadvantages are largely avoided through preliminary delignification by methods which leave the polysaccharides in practically their original state. Such delignified pulps are termed holocelluloses (1). While some depolymerization of the polysaccharides may occur, the degradative influences are held to a minimum in the production of holocellulose pulps. This paper presents some information on the preparation of cob holocellulose and some preliminary data on the properties of raw cobs and of cob holocellulose. Work on the isolation and further characterization of cob hemicellulose is in progress.

EXPERIMENTAL

Materials

A major part of the work was done upon commercially ground corn cobs which passed a 20-mesh screen. The pith and glumes had been removed for the most part by air separation.

¹ Journal Paper No. 341 of the Purdue University Agricultural Experiment Station.

For the starch determination, bright, clean, cobs were ground in a laboratory Wiley mill to pass a 20-mesh screen. Before grinding they were cracked and the pith removed.

Analytical Methods

Uronic acids were determined by the procedure of Whistler, Martin and Harris (2), acetyl values by the procedure of Freeman and Peterson (3) based on the method of Freudenberg and Harder (4), methoxyl by the method described by Niederl and Niederl (5), starch by the method of Sullivan (6), and lignin by a slight modification of the method of Ritter and Barbour (7). α -Cellulose (8), nitrogen (9), phosphorus (10), and ash were determined by the usual standard methods. Moisture content was determined by drying 0.5 g. samples for 4 hrs. at 100°C. in a vacuum oven. All analytical values reported in this manuscript are on a dry weight, ash-free basis.

Determination of the amounts of extractives removed by various organic solvents were made by Soxhlet extraction. Approximately 50 g. samples of ground cob were analyzed. At the end of 2, 8, 16, 24, 48, and 96 hrs., the extract was removed and replaced with a fresh quantity of solvent. The amount of extractives which were removed during each period was determined by evaporating the solvent from the solution in a weighed dish, drying the residue in a vacuum oven at 60°C., and weighing.

Preparation of Holocellulose

A. In Small Quantities. Approximately 30 g. of cobs, ground to 20-mesh size, were placed in a 1 l. round-bottom flask and 680 ml. of water were added. The mixture was mechanically stirred and heated to $74 \pm 1^\circ\text{C}$., at which temperature it was maintained by a thermostatically controlled water bath. Glacial acetic acid (2.5 ml.) was added, followed by the gradual addition of sodium chlorite (7.5 g.). This brought the pH of the mixture to 4.5. After 15 min., acetic acid and sodium chlorite were again added in the same quantities and this addition repeated at 15 min. intervals until 4 additions had been made. A glass tube placed in the neck of the flask permitted introduction of a constant stream of carbon dioxide to displace chlorine dioxide from the flask as soon as it was liberated. This precaution was taken to prevent the chlorine dioxide concentration in the air above the liquid from reaching that of an explosive mixture. When the carbon dioxide flushing was omitted, the chlorine dioxide formed in the flask would at times ignite spontaneously. The entire operation was carried out in a well-ventilated hood because of the high toxicity of the chlorine dioxide. During the early stages of the reaction the mixture foamed considerably, but the foaming was controlled by addition of 1 or 2 drops of octyl alcohol. After one hour, the mixture was rapidly cooled to about 20°C. and filtered through a filter cloth, washed with water until free of acid, and finally washed with acetone. The air-dried product was porous and almost white. The yield was 81.5%. Its lignin content was 0.4%.

B. In Larger Quantities. In a typical large scale preparation, 700 g. of ground cob containing 10% moisture was added to water in a 9 l. Pyrex serum bottle. The water level in the bottle was adjusted to within about 5 cm. of the neck. The mixture was stirred vigorously with a motor-driven Hershberg type stirrer. Four portions, each of 55 ml. of glacial acetic acid and 160 g. of sodium chlorite, were added successively.

Such large portions of sodium chlorite cannot be added instantaneously because of the excessive foaming induced. Therefore, each 160 g. portion was slowly poured in over a period of several minutes, and the total reaction period for the preparation was extended to 1.5 hrs. In all other respects the preparation was identical with the small scale preparation described above.

As indicated, both preparations (A) and (B) were performed at pH 4.5. It was observed that the efficiency of delignification decreased with decreasing temperature and with changes in pH to higher or lower values. At alkaline pH values excessive solubilization of holocellulose occurred, while at low pH values the rate of delignification decreased. For example, at pH 2 the lignin content of the holocellulose was reduced to only 6% in the 1 hr. treatment.

C. Other Delignification Methods. The activating action of chlorine was tested in one experiment. Chlorine gas was continuously bubbled through a mixture containing 1 g. of ground cob and 0.75 g. of sodium chlorite/24 ml. of water. The temperature was held at 40°C. and the reaction continued for 1 hr. These conditions reduced the lignin content to 9%.

Chlorine dioxide and chlorine, when bubbled for 1 hr. through a stirred mixture of ground cobs in water at pH 5-6 and at 75°C., produced a substance having a lignin content of 5.1%.

Extraction of Raw Cob and Cob Holocellulose with Potassium Hydroxide Solutions of Various Concentrations

To determine the alkali solubility of raw cob and of cob holocellulose the following experiments were performed: Approximately 30 g. samples of cob or cob holocellulose containing about 10% moisture were treated with 300 ml. of 1, 5, 10, or 17% potassium hydroxide solutions. Each suspension was stirred gently and maintained in an atmosphere of nitrogen at $25 \pm 1^\circ\text{C}$. for 24 hrs. The extracts from holocellulose were light yellow in color, while those from raw cob were quite dark.

The mixture was filtered on a filter cloth in each case, and the residue washed with dilute alkali and then with water, until no traces of color remained. The slightly alkaline residue was treated with a small amount of 10% acetic acid, washed until free of acid, and air dried.

The clear alkaline filtrate was acidified with 50% acetic acid to approximately pH 5. After allowing the mixture to stand for several hours, it was centrifuged in a super-centrifuge (40,000 r.p.m.). The precipitate, called the A-fraction after the nomenclature of O'Dwyer (11), was dehydrated by dispersing it in 500 ml. of 95% ethanol in a Waring Blendor and filtering. Care was taken that no air was drawn through the precipitate during the filtration. To insure the removal of water, the ethanol treatment was repeated 3 times. The precipitate was finally washed with ether to remove most of the ethanol, and immediately placed in a vacuum desiccator over calcium chloride. A white, finely-powdered product resulted.

The slightly opalescent centrifugate was poured into 3 times its volume of 95% ethanol with rapid stirring. The slightly yellow precipitate, called the B-fraction (11), was filtered and treated as described above.

DISCUSSION

A partial analysis of raw cobs is presented in Table I. These values are in approximate agreement with those obtained by Panasyuk (12) and those obtained by other workers.

TABLE I
Partial Analysis of Raw Corn Cobs and Cob Holocellulose

Analysis	Raw cobs per cent	Holocellulose per cent
Ash	1.32	1.40
Hexuronic acid anhydride	7.8	7.6
Acetyl	3.5	2.4
Methoxyl	2.0	0.5
Nitrogen	0.26	0.06
Phosphorus	0.024	—
Lignin	16.7	0.5
α -Cellulose	—	50.3 and 52.6 ^a
Starch	0.01 to 0.02	—

^a These data are average values of triplicate analyses made, respectively, on an air-dried and a 100°C. vacuum-dried sample.

The amount of extractives removed from the raw cob with different solvents are shown in Fig. 1. The action of these solvents is similar to

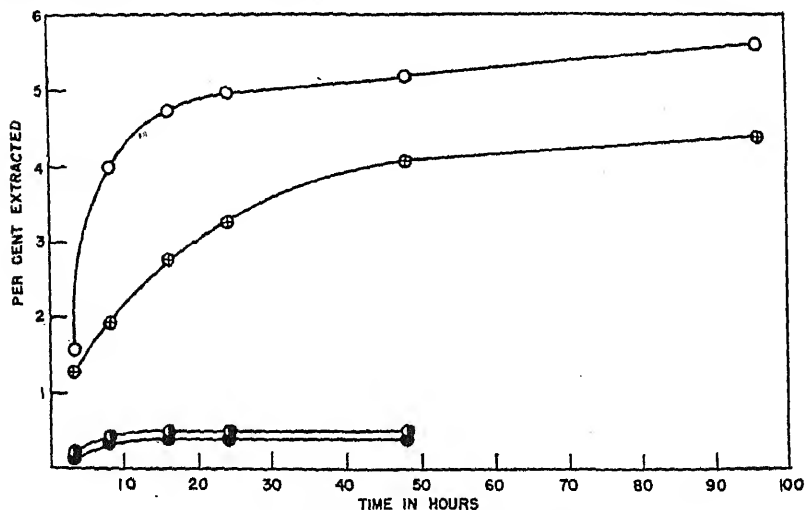


FIG. 1. Per cent extractives removed from raw cobs by
○ alcohol, ⊕ alcohol-benzene, ○ ether, ● benzene.

their action on woods. Ethanol, besides removing usual extractives, appears to dissolve a small quantity of low molecular weight carbohydrates. Hydrophobic agents such as benzene and ether only incompletely remove extractives. On the other hand, an azeotropic mixture consisting of 32 parts of ethanol and 68 parts of benzene appears to remove extractives without causing dissolution of carbohydrate.

Plant holocellulose is especially useful for the preparation of polysaccharides, since the polysaccharide material isolated from this material is almost free of lignin impurities. Holocellulose can most easily be prepared on a large scale by the sodium chlorite procedure of Wise, Murphy and D'Addieco (13). Cob holocellulose has recently been obtained in this manner by Bennett (14). However, the 3 hr. reaction period usually employed in the preparation of chlorite holocellulose is unnecessarily long for cobs, probably because cobs are more porous than wood. The rate of delignification is illustrated in Table II,

TABLE II
Holocellulose Yield and Lignin Content of Cobs Treated at Each of the Times Listed with 0.25 Part by Weight of Sodium Chlorite

Reaction period hrs.	Holocellulose yield per cent	Lignin content of holocellulose per cent
0	—	16.7
0.5	83.3	2.4
1	81.6	1.9
2	78.0	1.1
3	73.8	0.6
4	69.4	—
6	61.7	0.4
10	56.8	0.3

which shows holocellulose yield and lignin content of cobs treated at each of the times indicated with one-quarter part by weight of sodium chlorite and 2.5 ml. of glacial acetic acid. It is apparent that the major portion of the lignin is removed in the first half hour and that delignification is essentially complete in 3 hrs., or when a weight of sodium chlorite equal to that of raw cob has been added. However, since holocellulose is progressively lost during the reaction period, it is desirable to remove the lignin as rapidly as possible. This can be accomplished by increasing the rate of chlorite addition.

Results of several experiments indicate that lowest lignin values are obtained when chlorite is added at 15 min. intervals during the space

of 1 hr. A total of 1 g. of sodium chlorite is added and 24 ml. of water are employed for each g. of cob. By this process the yield of holocellulose is 81.5% and its lignin content only 0.4%. More rapid addition of chlorite produces holocellulose of higher lignin content. The color of the holocellulose obtained by chlorite treatment at 15 min. intervals remains a light straw-yellow. Slightly improved color results when delignification is performed in dark bottles or in the absence of light, since chlorine dioxide is rapidly decomposed by light. Longer periods of treatment produce a pure white product, but result in a loss of carbohydrate material. The use of ethanol-benzene extracted cobs also give slightly improved yields of holocellulose and a small improvement in color.

While cobs ground to 40-mesh size are used in most instances, no significant change in lignin content for the holocellulose is observed with 20- or 80-mesh cobs or with coarse-ground particles whose dimensions are approximately 3×1 mm.

When the amount of water is reduced to one-half, or 12 ml. for each g. of cob, the slurry is somewhat harder to stir. If the water is reduced to 6 ml. for each g. of cob, stirring becomes very difficult and the lignin content of the holocellulose is found to be above 3%. Consequently, 12-24 ml. of water/g. of cob appears to be most satisfactory.

In these experiments, as well as those of other workers, approximately equal quantities of sodium chlorite and plant material are used for the preparation of holocellulose. Several experiments have been made to determine whether it might be possible to reduce this large reagent requirement. Russell (15), and White and Vincent (16, 17), describe the bleaching of pulp by sodium chlorite activated with acid, chlorine, or hypochlorite. As indicated in the "Experimental" part, acid activation of chlorite at pH 4.5 appears to be better for the preparation of holocellulose than other types of activation. In the several experiments tried it has not been found possible to replace a part of the chlorite with chlorine. Replacement of a part of the chlorite with hypochlorite is unsatisfactory because, under the high pH condition required, a considerable dissolution of hemicellulose occurs. Thus, at pH 8.5-9.5, all of the B-fraction and a part of the A-fraction are dissolved. A mixture of chlorine dioxide and chlorine gas produces high delignification but not as much as does sodium chlorite at pH 4.5.

The extent to which hemicelluloses are removed from raw cob and cob holocellulose by extraction with sodium hydroxide solutions of

different strengths are shown in Figs. 2 and 3. These figures indicate that the solubility of hemicellulose increases with increasing alkali concentration until the alkali concentration reaches 10%. Solutions of higher alkali concentration extract only a small additional amount of carbohydrate. Since these hemicelluloses are not quantitatively removed by a solution of low alkali concentration, they appear to have different solubility characteristics than those of wheat straw (18).

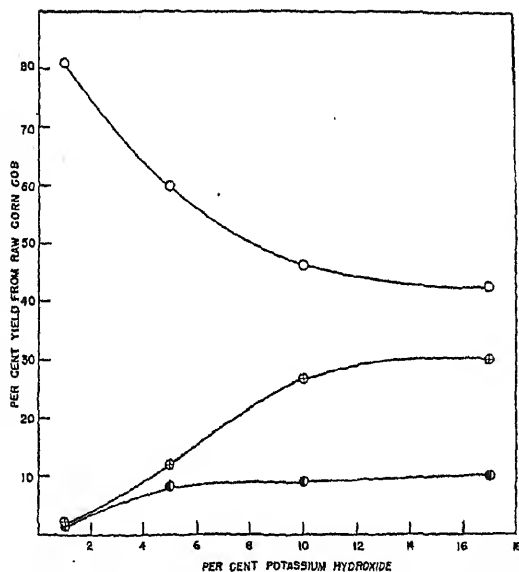


FIG. 2. Yield of A and B hemicellulose and residue obtained by alkali extraction of raw corn cob. ○ Residue, □ A-fraction, ● B-fraction.

A comparison of Graphs 2 and 3 indicates that in holocellulose the hemicellulose B is completely soluble in 0.5% alkali, whereas in the raw cob the solubility of B increases as the concentration of alkali increased from 1 to 5%. Thus, hemicellulose B is made more available to extraction by the conversion of cobs to holocellulose.

The alkali extract of raw cobs on acidification yields an A fraction containing appreciable quantities of lignin. This accounts for the higher yield of the A fraction obtained from the raw cob.

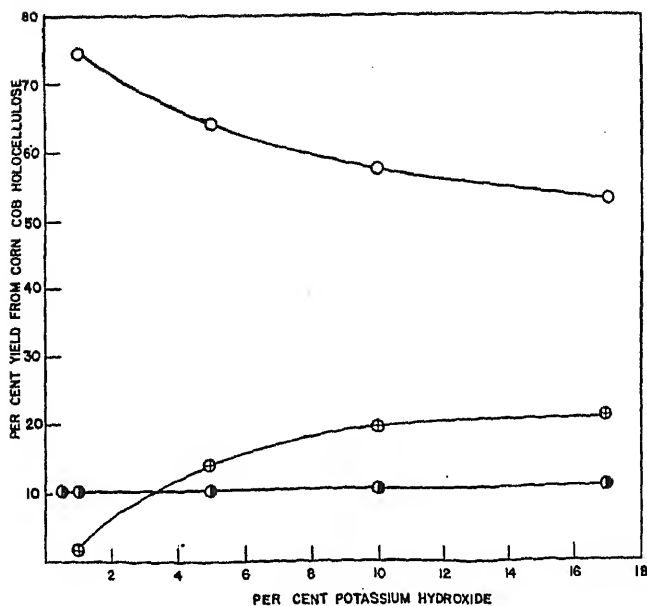


FIG. 3. Yield of A and B hemicellulose and residue obtained by alkali extraction of corn cob holocellulose. \circ Residue, \oplus A-fraction, \bullet B-fraction.

ACKNOWLEDGMENT

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SUMMARY

Corn cob holocellulose can be prepared in 1 hr. by a slight modification of the usual sodium chlorite procedure. A partial analysis is given of raw cob and its derived holocellulose.

The total amount of hemicellulose extracted from raw cob or cob holocellulose by potassium hydroxide solution at 25°C. increases with increasing alkali concentration up to 10%, but at a concentration greater than this only small additional amounts of hemicelluloses are extracted.

Hemicellulose B-fraction of raw cob is insoluble but the hemicellulose B of cob holocellulose is soluble in 0.5% potassium hydroxide solution

at 25°C. Hence, the B-fraction is freed during the delignification process.

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Amperometric Titration of Sulfhydryl Groups in Amino Acids and Proteins

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INTRODUCTION

A variety of methods have been used for the determination of sulfhydryl groups in compounds of biological importance, but only some of these are applicable to the estimation of sulfhydryl groups in proteins (1, 2, 3, 4, 5, 6).

It is the purpose of this paper to show that the amperometric procedure described for the determination of mercaptans by Kolthoff and Harris (7) can be applied to the determination of sulfhydryl groups in cysteine and glutathione, as well as in certain proteins.

EXPERIMENTAL

Principle of the Method

When a mercaptan in alcoholic solution (or aqueous alcoholic solution) is titrated with aqueous silver nitrate solution at a rotating platinum electrode against a suitable reference electrode, the insoluble silver mercaptide is precipitated. A negligible current flows until there is an excess of silver ions in solution. At this point the diffusion current of silver ions to the rotating platinum electrode rises sharply and in proportion to the concentration of these ions in the solution. The end-point of the titration is obtained graphically by plotting current readings against volume of silver nitrate solution added, and noting the point of intersection of the two straight lines (Figs. 1 and 2). Kolthoff and Harris also applied this method to sulfhydryl determinations in the presence of chloride ions by titrating in ammoniacal solution and using a mercury/mercuric iodide electrode to complete the circuit. Under these latter conditions satu-

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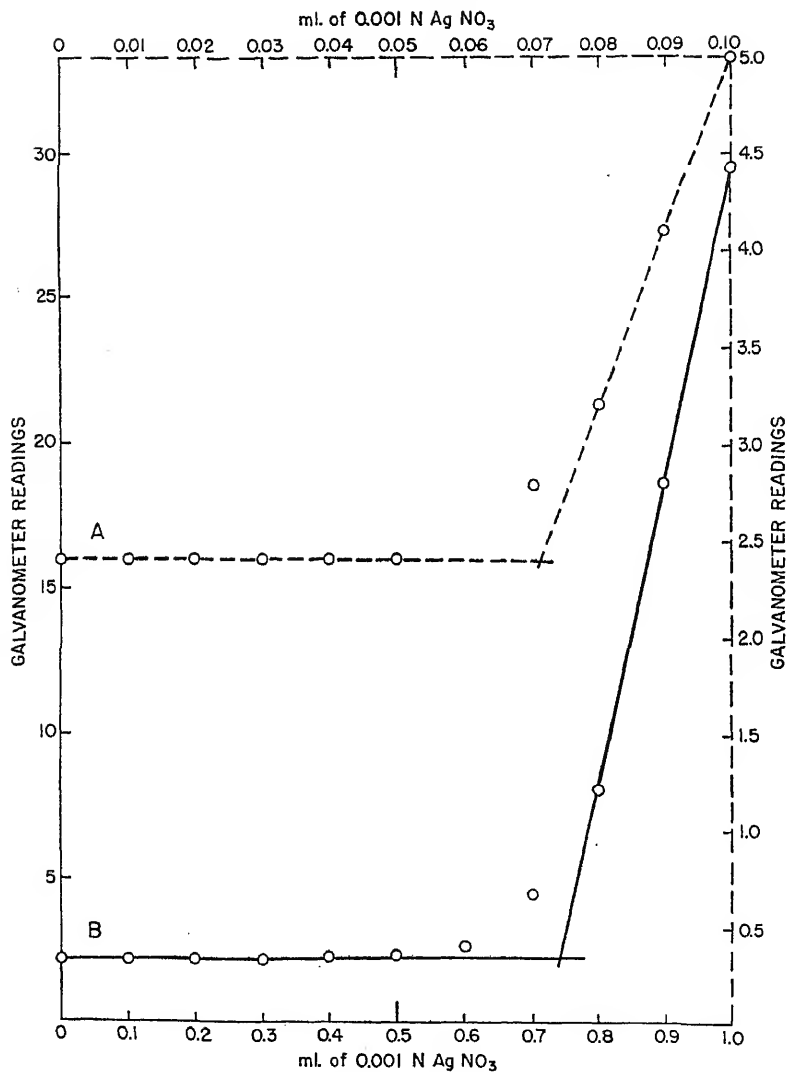


FIG. 1. Titration of dialyzed serum. Curve A: 0.3 ml. serum used for titration; Curve B: 3.0 ml. serum used for titration.

rated potassium chloride was used as a salt bridge and ammonium nitrate as a supporting electrolyte. All the work presented in this paper was carried out in this system.

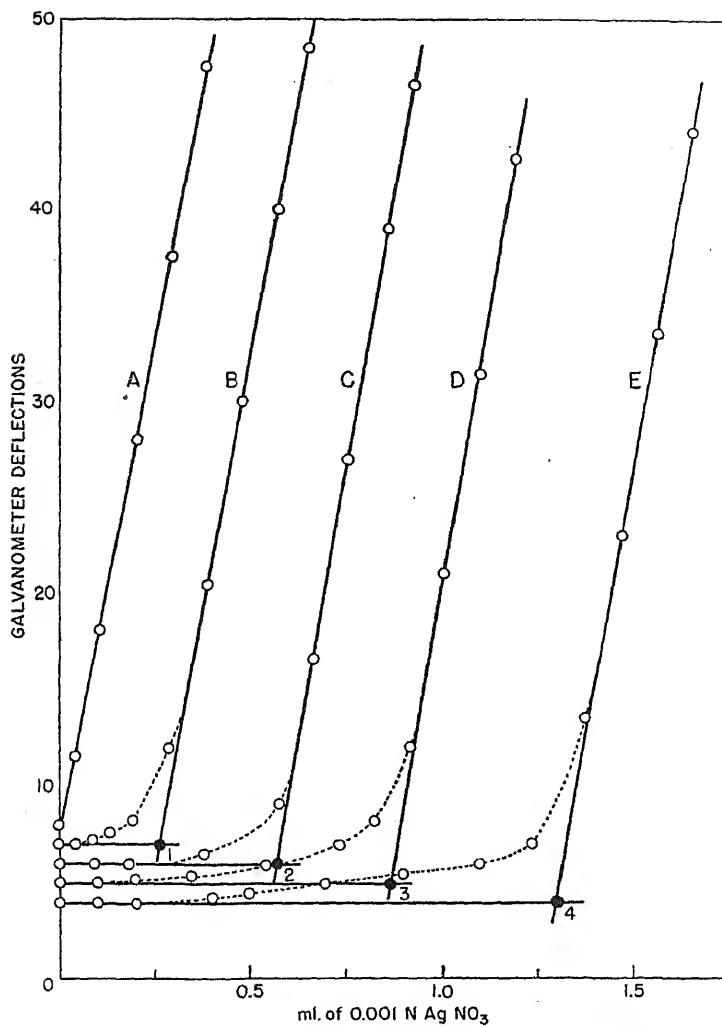


FIG. 2. Titration of 2 ml. portions of a 5.67% solution of bovine serum albumin. Curves A, B, C, D, and E represent titrations after the addition of 1.51, 1.08, 0.75, 0.43, and 0.00 ml. of 0.001 *N* *p*-chloromercuribenzoic acid, respectively, and points 1, 2, 3 and 4 represent the end points of curves B, C, D, and E, respectively.

Apparatus

The apparatus and procedure used were essentially the same as those described by Kolthoff and Harris. However, since we assayed quantities 10–100 times smaller than

those estimated by these authors, 0.001 *N*, instead of 0.005 *N*, silver nitrate was used as the titrating reagent, a more sensitive galvanometer (G.E. 320/235/81) was used, and microburettes, including a Rehburg burette for some of the titrations, were employed.

Experimental Substances

1. *Thioglycollic Acid, Cysteine, Ergothioneine, and Glutathione.* These were diluted from stock solutions to 30 ml. with 95% ethanol and the solutions made 0.05 *M* in ammonium nitrate and 0.25 *M* in ammonia. The final ethanol concentration was about 90%. They were then titrated with 0.001 *N* aqueous silver nitrate solution.

2. *Proteins.* These were titrated with the 0.001 *N* silver nitrate solution under three different sets of conditions:

(a) *Titration in Solutions of Varying Alcohol Concentrations.* Thirty ml. of varying mixtures of water and ethanol (0–50%) (95% in the case of zein) were prepared and cooled to room temperature, ammonia and ammonium nitrate were added to give the indicated concentrations, and finally the protein solution was stirred in dropwise, care being taken to prevent precipitation.

(b) *Titration in Guanidine Hydrochloride Solution.* Denaturation with this reagent was carried out according to the method of Greenstein (13, cf. 8). The denatured material was then diluted to 30 ml. with the addition of the indicated quantities of ammonia and ammonium nitrate.

(c) *Titration in Duponol P.C. (cf. 9) Solution.* This denaturation was carried out as described by Anson (3) and the solutions were then treated as described under (b) above.

3. *Sera.* Ten ml. samples of human serum were dialyzed against 100 volumes of distilled water at 4°C. for 48 hrs. and then for 1 hr. against 100 volumes of normal saline. The increase in volume after dialysis was measured so that allowance could be made in the calculation of the "combined cysteine"/100 ml. original serum (cf. Table IV). Three ml. of the dialyzate were diluted with water, ammonia and ammonium nitrate were added in the usual amounts, followed by a volume of alcohol insufficient to cause any turbidity (final volume 30 ml.). By using a Rehburg microburette it was possible to titrate as little as 0.3 ml. of dialyzed serum, as was done with some samples. All the serum determinations were carried out in triplicate.

Use of Standard p-Chloromercuribenzoate (10)

Samples of all the substances tested were treated with an excess of this reagent to observe the effect on the silver-titratable sulfhydryl groups. In the case of the crystalline proteins, moreover, graded amounts of this reagent were also added and the residual sulfhydryl groups estimated amperometrically with silver nitrate (cf. Table III).

Titration of Protein Sulfhydryl Groups with Standard p-Chloromercuribenzoate, Using Sodium Nitroprusside as Indicator (3)

One ml. samples of the respective proteins were denatured with guanidine hydrochloride. They were then cooled in ice, varying amounts of a standard solution of

ammonium *p*-chloromercuribenzoate were added, followed by the addition of dilute sodium nitroprusside solution and concentrated ammonia. Arrival at the end-point in this discontinuous titration was indicated by the sample which first failed to show any color.

The nitrogen content of the protein and serum solutions used was determined by a semimicro Kjeldahl procedure and the sulphhydryl content was calculated on this basis.

Materials

Thioglycollic acid—British Drug Houses, Ltd. (redistilled).

Cysteine hydrochloride—Eastman Kodak Co. (analyzed sample).

Glutathione—Schwartz Laboratories (analyzed sample).

Ergothioneine monohydrochloride dihydrate—sample obtained by the courtesy of Dr. A. Lawson.

All other aminoacids used—Eastman Kodak Co.

Crystalline egg albumin—prepared by the method of Kekwick and Cannan (11).

Crystalline bovine serum albumin—Armour (Fraction V).

Crystalline human serum albumin—sample from the laboratory of Dr. E. J. Cohn (Fraction V, run 164), donated by Dr. M. Rosenfeld.

Gelatin—commercial sample, 87% protein.

Zein—Pfanstiehl, 90% protein.

p-Chloromercuribenzoic acid—one sample was obtained by synthesis (12) and for an analyzed sample we are indebted to Dr. L. Hellerman.

Guanidine Hydrochloride—highly purified sample.

Duponol P.C.—high grade commercial sample.

RESULTS

1. Simple Sulphydryl Compounds

These results are summarized in Table I. From these data both the accuracy and the reproducibility of the method may be judged. It is evident that only the ergothioneine results show a large but rather constant deviation from the theoretical value. The possibility that the high values are due to a reaction of the silver-ammine ion with the imidazole ring is made unlikely by the negative results obtained with histidine.

The smallest quantities of mercaptan sulfur which we determined were about 0.03 mg. When it was attempted to determine quantities as small as 0.003 mg., the accuracy of the method decreased considerably.

All the compounds listed were also titrated after treatment with an excess of 0.001 *N* *p*-chloromercuribenzoate and, in each case, the silver titration was found to be zero except in the case of ergothioneine.

TABLE I
Amperometric Titration of Simple Sulfhydryl Compounds

Compound	No. of determinations	Mg. present	Mg. found	Per cent recovery
Thioglycollic acid	5	0.46	0.45±0.02	98
Thioglycollic acid	1	0.37	0.37	100
Thioglycollic acid	2	0.092	0.088±0.05	96
Thioglycollic acid ^a	2	0.46	0.46±0.02	100
Cysteine hydrochloride	3	0.162	0.166±0.002	102.5
Glutathione	3	0.259	0.256±0.002	99
Glutathione	1	0.518	0.520	100.4
Glutathione	1	1.036	0.983	95
Glutathione	1	1.554	1.510	97
Ergothioneine hydrochloride dihydrate	5	0.500	0.559±0.017	112

^a Titrations were carried out under nitrogen.

2. Crystalline Proteins

The results are summarized in Table II. In proteins—and also in sera—quantities of mercaptan sulfur as small as 0.002 mg. could be accurately determined (see Fig. 1). In agreement with the observations of other workers (14) it was found impossible to secure combination of potentially available sulfhydryl groups of egg albumin in aqueous solution, although a slow appearance of sulfhydryl groups was noted. A steady and substantially linear increase in titratable sulfhydryl groups was observed with increasing alcohol concentration, probably as a result of progressive denaturation. This was not so in the case of the serum albumins. It can also be seen that the results in guanidine hydrochloride solution were always lower than those obtained under other conditions. This may have been caused by the insolubility of the denatured albumin, and also by the effect of the high ionic strength of the solution on the accuracy of the electrometric titration. The titrations with *p*-chloromercuribenzoate, using sodium nitroprusside as an indicator, gave somewhat lower results than those obtained by the amperometric method. For egg albumin the sulfhydryl values (expressed in g. cysteine/100 g. protein) in the higher alcohol concentrations are in good agreement with those reported by other workers [Greenstein—1.28% (13), Hellerman—1.29% (8), Anson—1.24% (3),

TABLE II

Sulphydryl Content of Crystalline Proteins

All determinations were carried out by the amperometric silver titration unless otherwise stated

Condition of assay	Egg albumin ^a		Bovine serum albumin ^b		Human serum albumin ^c	
	No. of experiments	—SH groups expressed as g. of cysteine/100 g. protein	No. of experiments	—SH groups expressed as g. of cysteine/100 g. protein	No. of experiments	—SH groups expressed as g. of cysteine/100 g. protein
In water	—	—	2	0.14±0.00	1	0.075
In 20% ethanol	2	0.90±0.03	—	—	—	—
In 32% ethanol	5	1.08±0.04	4	0.14±0.00	6	0.072±0.001
In 40% ethanol	2	1.16±0.00	—	—	—	—
In 50% ethanol	4	1.34±0.06	1	0.15	1	0.072
In 32% ethanol after denaturation with guanidine HCl	2	0.85±0.03	—	—	—	—
In water after denaturation with guanidine HCl	2	0.80±0.01	2	0.12±0.00	2	0.054±0.002
In water after denaturation with guanidine HCl ^d		0.96		0.13		0.043
In water after denaturation with Duponol P.C.	2	0.90±0.00	—	—	2	0.076±0.000

^a One ml. of a 2.23% solution was used for each titration.

^b Two ml. of a 5.67% solution were used for each titration.

^c Two ml. of a 5.49% solution were used for each titration.

^d These estimations were carried out as an independent check using *p*-chloromercuribenzoate (0.001 *N*) as titrating agent and sodium nitroprusside as an indicator.

Mirsky—0.96% (4)]. For bovine serum albumin the only available value is that reported by Greenstein (15), *i.e.*, 0.34% which is considerably higher than our results, but which was obtained on a sample prepared by another method. In view of the differing conditions of assay and of possible differences in the character of the samples examined, stress should not now be laid upon the apparent discrepancy.

Treatment of the protein solutions with an excess of *p*-chloromercuribenzoate resulted in complete disappearance of the silver-titratable

sulfhydryl groups under all conditions used. Moreover, the addition of graded amounts of this reagent resulted in a stoichiometric removal of the silver-titratable sulfhydryl groups (*cf.* Table III). This is regarded as adequate proof that *p*-chloromercuribenzoic acid and the silver-ammine ion react with the same groups of the protein under the conditions employed.

TABLE III
Amperometric Determination of Residual Sulfhydryl Groups after Treatment with Varying Amounts of p-Chloromercuribenzoate
All the estimations were carried out in 32% ethanol solution

	0.001 <i>N</i> <i>p</i> -chloromer- curibenzoate added	0.001 <i>N</i> AgNO ₃		—SH groups expressed as g. of cysteine/ 100 g. protein	
		Calculated	Found	Calculated	Found
	<i>ml.</i>	<i>ml.</i>	<i>ml.</i>		
Crystalline egg albumin	0.00	—	1.99	—	1.08
	0.65	1.34	1.25	0.73	0.68
	1.08	0.91	0.86	0.49	0.47
	1.62	0.37	0.50	0.20	0.27
	2.16	0.00	0.00	0.00	0.00
Crystalline bovine serum albumin	0.00	—	1.30	—	0.14
	0.43	0.87	0.88	0.093	0.094
	0.75	0.55	0.58	0.059	0.062
	1.08	0.22	0.25	0.024	0.027
	1.51	0.00	0.00	0.000	0.000
Crystalline human serum albumin	0.00	—	0.43	—	0.071
	0.10	0.33	0.35	0.054	0.057
	0.20	0.23	0.21	0.037	0.034
	0.30	0.13	0.13	0.021	0.021
	0.50	0.00	0.00	0.000	0.000

In order to test further the specificity of the method, the following amino acids were titrated in 32% ethanol in amounts 10 times greater than the quantities used for the titration of cysteine: histidine, arginine, methionine, tyrosine, tryptophan, glutamic acid, serine, and proline. The titration for all of these compounds was found to be zero. In addition, gelatin and zein were tested as examples of proteins which do not contain appreciable amounts of sulfhydryl groups. In both cases, 2 ml. portions of a 1% solution gave no evidence of titratable sulfhydryl groups.

In view of the action of ammonium ions on the sulfhydryl groups of myosin reported by some investigators (2, 16), and a similar effect on some possible protein models (17), it should be borne in mind that all our determinations were carried out in the presence of considerable concentrations of ammonium ions.

3. Sera

Dialyzed² sera were used, in order to remove possible interfering substances. Very reproducible results were obtained, some of which are presented in Table IV. The applicability of the method, as applied

TABLE IV
Amperometric Titration of Dialyzed Human Sera

Diagnosis	—SH groups expressed as (mg. cysteine /mg. N) $\times 10^{-3}$		—SH groups expressed as mg. cysteine /100 ml. orig- inal serum	
Normal	4.65		6.53	
Normal	4.68		5.61	
Normal	3.48		4.19	
Normal	4.60		5.66	
Normal	5.18	4.89 ^a	5.84	5.52 ^a
Normal	5.28		6.13	
Syphilis	4.80		5.08	
Amoebic hepatitis	4.73		5.48	
Uterine bleeding	5.07		6.41	
Rheumatic fever	4.90		5.87	
Coronary thrombosis	4.11		4.36	
Amenorrhea and bacilluria	4.22 ^a		5.49 ^a	
Diabetes	3.85	3.97 ^a	4.55	4.70 ^a
Rheumatoid arthritis	3.42		3.95	
Carcinoma of stomach	4.05		4.07	
Carcinoma of bronchus	3.28		—	

^a Determinations carried out on 0.3 ml. of dialyzed serum.

to sera, was checked by adding a slight excess of *p*-chloromercuribenzoate to the sera. This reduced the silver titration to zero in every case.

p-Chloromercuribenzoic acid was chosen as a reagent for testing the specificity of this method throughout this investigation in view of the advantages of compounds of this class in studies of this kind. Olcott

² Subsequent work has shown, however, that dialysis is unnecessary, since the results obtained with dialyzed and undialyzed sera proved to be identical.

and Fraenkel-Conrat (18), in a recent review, have summarized the advantages attending the use of such compounds:

"(1) They have a great affinity for —SH groups and are thus effective under mild conditions and in low concentrations; (2) they react with single —SH groups; (3) they react with —SH groups that appear masked to many oxidizing agents; (4) they appear highly selective in combining with no protein group other than —SH; and (5) their combination with —SH groups is reversible by the addition of an excess of a simple mercaptan."

ACKNOWLEDGMENTS

It is a pleasure to be able to express our gratitude to Dr. W. M. Clark and to Dr. L. Hellerman for the facilities which they generously placed at our disposal to complete this work and for valuable help and advice.

SUMMARY

(1) An amperometric method for the determination of sulfhydryl groups in aminoacids, peptides and proteins is described.

(2) Results of titrations of simple sulfhydryl compounds, crystalline proteins and dialysed human sera are presented, and the accuracy and specificity of the method are discussed.

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Ascorbic Acid Oxidation and Browning in Apple Tissue Extracts¹

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INTRODUCTION

The mechanism of ascorbic acid oxidation in fruits is an interesting field of study from both a practical and an academic standpoint. As will be shown later, ascorbic acid oxidation is intimately associated with darkening of cut or injured fruit tissue exposed to air. As a typical fruit oxidation system, that of apples was selected for a rather detailed study. The complete enzyme-substrate system in apple juice, and also the separate enzymes with synthetic substrates, were studied. The results should be applicable in general to many other fruits and some vegetables with similar enzyme systems.

Ascorbic acid is known to be oxidizable by 4 systems: direct oxidation by ascorbic acid oxidase (ascorbbase), originally suspected by Szent-Györgyi (1) and definitely proven by Tauber and Kleiner (2), Hopkins and Morgan (3), Johnson and Zilva (4), and others; by *o*-quinones arising from activity of peroxidase, originally postulated by Szent-Györgyi (5) and shown by Tauber (6) and others, or by flavones in presence of peroxidase and peroxide, (7); by *o*-quinones formed by catechol plus polyphenol oxidase (polyphenolase), Johnson and Zilva (4), Keilin and Mann (8), and others; and by the cytochrome system (9). Another plant enzyme, laccase, evidently is also able to oxidize ascorbic acid through the medium of phenolic compounds, (10). Comparative studies on two plant systems have shown that, in the presence of sufficient polyphenols, the rate of oxidation of ascorbic acid by the phenolases is much more rapid than by ascorbic acid oxidase (10, 11).

Ascorbbase has apparently not been found in fruits, judging from a survey of the literature, although it is present in many vegetables. Wachholder (12) reported a small amount present in plums but not in other fruits, but his method of measuring activity was not very

¹ Based upon data presented by senior author in PhD thesis, University of California, June 1944.

trustworthy because of much autoxidation of ascorbic acid. The complete cytochrome system has not been reported in fruit and is not an important terminal oxidase system in plants (13). Laccase is merely a particular kind of polyphenol oxidase found in the latex of the lac tree and need not be considered separately here. The absence of ascorbase and the presence of both polyphenolase and peroxidase in apple juice was proved by the experiments described below and our study was limited to the latter two enzyme systems.

EXPERIMENTAL

Investigations with Purified Ascorbase, Peroxidase and Polyphenolase

To develop reliable techniques for the measurement of the activity of the above enzymes in fruit tissue and for determination of their relative contribution to the overall oxidation of ascorbic acid, purified ascorbic acid oxidase (ascorbase) was prepared from yellow crookneck summer squash, purified peroxidase from rutabagas, and purified polyphenol oxidase (polyphenolase) from apples.

Extraction of all the enzymes was accomplished in a Waring Blendor. By blending the plant material for 2 or 3 mins. in ice water, and filtering, crude enzyme solutions can be prepared very quickly, and the loss in activity during blending appears to be low—no greater, and probably less, than by grinding in a mortar with sand or similar procedures.

Acetone precipitation of the enzymes was employed to a large extent in the purification procedures for all 3 enzymes. This method has usually been avoided by other workers because acetone caused much denaturation of the enzymes. However, the temperature of the acetone was usually between 0° and 25°C. in these cases. It was found that, by precipitating the enzymes from cold solutions with acetone at -23°C. (-10°F.), better yields could be obtained in most cases than by salting out with ammonium sulfate. The yields with acetone varied ordinarily between 70 and 100%. Also, acetone yielded more compact and faster-settling precipitates beside eliminating the necessity for dialysis in most cases. This is important in the case of polyphenolase as this enzyme is very delicate, sometimes losing half its activity on dialysis at 0°C.

By this method the separation of all naturally occurring phenolic compounds was readily accomplished. Removal of peroxidase from the other enzymes is especially difficult because it is more stable, in general, and has about the same precipitation characteristics. This makes separation by fractional precipitation with salts or organic solvents rather impracticable. To remove peroxidase, advantage was taken of the fact that it is much more subject to surface denaturation in dilute solution than the other enzymes. Thus, by filtering the preparations several times during purification, peroxidase content was gradually reduced to practically zero without an accompanying large loss of the other enzymes.

The ascorbase was prepared by blending 300 g. of squash slices with 600 ml. of ice cold tap water, filtering through coarse Celite filter aid, and serially precipitating with cold acetone, at first at 60% concentration and an additional 3 times at 50%. The final

flocculent greenish-white precipitate was dissolved in 11 ml. of water and represented a concentration on the dry weight basis of 1076 times for ascorbase and 0.042 times for peroxidase, *i.e.*, a removal of all but a trace of the original peroxidase. The residual peroxidase activity is easily measurable in concentrated solution but not in the 1000:1 dilution used in measuring ascorbase activity. This solution was frozen in test tubes in 0.5 ml. portions at -23°C . in which state it retained its activity for several months.

Rutabaga peroxidase was prepared by a similar method, but the cold water extraction led to low recovery and difficulty in separating it from inactive material. Extraction with a buffer solution at a temperature high enough to coagulate much of the inactive protein and other enzymes without too much inactivation of peroxidase was found to be superior. 1200 g. of rutabagas were blended with twice their weight of hot (65°C .) 0.025 *M* acetate buffer of pH 5.0 for 3 mins. and filtered through Celite filter-aid, yielding 2750 ml. of slightly turbid, pale yellow filtrate. This solution was cooled to its freezing point and the enzyme precipitated with 2 volumes of acetone cooled to -23°C . This precipitation was then repeated 3 times; the precipitate was allowed to stand for 2 hrs. in 50% acetone before centrifugation. The final precipitate was dissolved in 0.01 *M* acetate buffer to yield 17 ml. of clear very pale tan solution representing a 445-fold concentration of peroxidase on the dry basis. This solution could be stored at 0°C . without any appreciable loss of activity on prolonged storage. It was diluted 2500:1 before use; in the dilute form it was unstable at room temperature.

Apple polyphenolase was found to be firmly adsorbed on particles of apple tissue, so that it was impossible to obtain a clear solution containing the enzyme from the pressed juice. Advantage was taken of the fact that apple polyphenolase was stable in alkaline solutions and was not adsorbed to the insoluble material from them. Extraction with 2% Na_2CO_3 gave a clear, active extract after filtration, having a pH of about 11.3. 800 g. of apple tissue were blended 2 mins. with 1600 ml. ice cold 2% Na_2CO_3 , filtered through Celite filter-aid, precipitated with 2 volumes of acetone precooled to -23°C ., and filtered immediately through a milk filter disc. The resulting precipitate was dissolved in ice water, refiltered, and pectinous material was precipitated with cold acetone at 29% final concentration. The supernatant was filtered, the enzyme precipitated with 3 volumes of cold acetone, and redissolved in ice water. The solution was then treated with Amberlite IR-100 cation exchanger to a pH of 5.0 filtered, and reprecipitated after solution, first with 4 volumes and then with 5 volumes of cold acetone. In the last treatment boric acid was added to obtain a precipitate. The dark red-brown precipitate which resulted was centrifuged out, redissolved in cold distilled water, and treated with a mixture of Amberlite cation and anion exchangers to pH 6.4. The solution was then filtered through sintered glass, yielding 13 ml. of solution representing a concentration, on the dry weight basis, of about 600 times the activity in apple tissue.

The solution was pale yellowish-brown in acid solution, agreeing with the findings of Kubowitz, (14) Keilin and Mann (15), and Sreerangachar (16), and pale pink or reddish in alkaline solution, as was also found by Sreerangachar. Freezing in dry ice and acetone caused no loss, but subsequent storage at -30°F . caused a loss of 80% of the activity in 3 months. Drying the frozen solution (lyophilizing) rendered the enzyme completely insoluble. The loss in solution at 0°C . is about as low as in frozen,

storage. The preparation still contained some peroxidase activity, but this did not amount to more than 5% of the polyphenolase activity, which is about the experimental error in measuring the enzyme activity.

The apple polyphenolase appears to be quite similar to the tea leaf polyphenolase of Sreerangachar in respect to purity and instability. With both of these preparations there was much loss in activity on dialysis. The use of synthetic resin ion exchangers to remove salts, in place of dialysis, has the advantage of being very rapid and not causing an appreciable loss in activity. It would seem from the work of Keilin and Mann that their polyphenolase preparation from mushrooms was more stable than the apple or tea leaf preparations; they used many dialyses in the course of purification without mentioning consequent losses in activity.

The above enzyme preparations were used in our studies of the effect of several environmental conditions on the kinetics of enzymic oxidation of ascorbic acid.

MEASUREMENT OF ENZYME ACTIVITIES

The methods described below were selected or developed for rapidity and accuracy.

The method developed for measuring *ascorbase* activity is as follows:

Twenty ml. of 0.1 *M* oxalate-0.1 *M* phosphate buffer of pH 6.0, containing approximately 1 mg. of ascorbic acid, is pipetted into a 100 ml. volumetric flask which has a glass stopper attached by a stopper tie. The flask is floated on the surface of a water bath at 25°C., the glass stopper hanging over the edge to hold the flask in place. When temperature equilibrium has been attained, a suitable quantity of enzyme, usually 1 or 2 ml. of solution, is added by means of a calibrated, rapid delivery pipette. The flask is shaken and refloated. No further agitation or oxygenation is necessary. The reaction is allowed to proceed for a measured interval, usually 2 mins., after which it is stopped by adding approximately 3 ml. of *M* oxalic acid by blowing it from a large bore pipette. The flask is made to volume with distilled water, which brings both the ascorbic acid and oxalic acid concentrations within the correct range for measuring ascorbic acid (17), and the residual ascorbic acid is measured. A zero-time blank to measure the exact initial amount of ascorbic acid is run by mixing the ascorbic acid and enzyme and then immediately adding oxalic acid. This takes 4 or 5 secs., which is added to the reaction times. This method was found to give consistently better zero points than by adding acid before adding the enzyme, the method originally used.

The residual ascorbic acid was measured colorimetrically by a slight modification of the method of Loeffler and Ponting (18). The accuracy was brought to within ± 0.01 mg. ascorbic acid/100 ml., that is ± 0.01 mg. in the reaction mixture, by using 4 ml. or more of the ascorbic acid solution plus 9 ml. of 2,6-dichlorobenzene-indophenol dye instead of 1 ml. of solution plus 9 ml. of dye as used in the published method. This usually gave an accuracy in the enzyme measurement of 2-10%, depending on the amount of ascorbic acid oxidized. A quantity of enzyme should be used such that not more than one-third, or at most one-half, of the ascorbic acid

present will be oxidized. If this procedure is followed, the reaction is of zero order for several minutes, and the slope of the rate curve is proportional to the amount of enzyme.

The pH value of 6.0 is the optimum for ascorbase activity in oxalate-phosphate buffer and was used for that reason, although the results of numerous investigators show that the optimum may range from a pH of 5.3 to over 6, depending upon the type of buffer used, and perhaps also upon the source of the enzyme. Oxalate-phosphate buffer was used for two reasons: first, to eliminate nonenzymic or autoxidation during the test period, which has often been the cause of untrustworthy results in the past; and second, to increase the buffer capacity over that of oxalate alone, which is low at pH 6.0. Oxalate has the property of completely preventing nonenzymic oxidation for at least 20 mins. It is especially effective in preventing oxidation catalyzed by inorganic copper, while at the same time not affecting enzymic oxidation to a great extent as shown by Krishnamurthy (19), Giri (20), and Ponting (17). Orthophosphate gives a fairly high buffer capacity at pH 6; pyrophosphate was tried because, according to Krishnamurthy and Giri (21), it does not inhibit the enzymic oxidation, but it was found to have too low a buffer capacity. Orthophosphate by itself allows very rapid autoxidation of ascorbic acid (25% in 10 mins. at 25°C.), but by adding oxalate to a concentration of 0.1 *M* the autoxidation is eliminated, except above 50°C. There is some indication of inhibition of ascorbase by phosphate, but the effect is slight. A citrate-phosphate buffer has been used successfully by several investigators, but Lineweaver (22) has found that oxalate added to a medium containing citrate has an additional effect in preventing oxidation catalyzed by copper and other metals.

A rapid colorimetric method was usually used for measuring *peroxidase* activity, based on the rate of color formation when peroxidase is mixed with hydrogen peroxide and guaiacol. It is a modification of the method developed by Masure and Campbell (23), and is similar to the method used by Hassein and Cruess (24) except for the time factor. The latter authors employed a reaction time of 1 hr. which was found to be long enough to allow considerable enzyme inactivation.

The method is as follows: One ml. of 0.5% (0.04 *M*) aqueous guaiacol and 1 ml. of 0.1 *N* hydrogen peroxide are added to 20 ml. of 0.01 *M* acetate buffer at pH 5.0 and 25°C. in an Evelyn colorimeter tube. Then 1 ml. of enzyme solution is added (or more enzyme solution with correspondingly less buffer), the tube is shaken and placed in the colorimeter. Readings are taken at intervals of 5 to 30 secs., depending on the rate of the reaction, using a 420 m μ filter. The values of $2\text{-log } T$ (T = per cent of light transmission) are obtained from the Evelyn chart and plotted against time. The slope of the straight line obtained, *i.e.*, the rate, was found to be proportional to the enzyme concentration. The activity is expressed in $\Delta\text{log } T$ units/min. The temperature is not controlled while the tube is in the colorimeter, but does not change enough to cause a measurable error.

This method is very sensitive, requires only a few minutes for an activity measurement, and has the advantage of measuring the initial rate of oxidation; this is important because the rate sometimes falls off after a few minutes. The reaction is of zero order.

The line obtained by plotting $2\text{-log } T$ against time is not straight at the beginning when readings are started immediately after mixing enzyme and substrate. The lag

period lasts from a few seconds, with a strong enzyme solution, to about 3 or 4 mins., with a very weak enzyme solution, after which the line is straight for several minutes. Therefore, with slow reactions it is not necessary to start readings until about 2 or 3 mins. after mixing the enzyme and substrate.

The ^{accuracy}precision of this method is well within 5%. A comparison with the method of Balls and Hale (25) indicated that the colorimetric method could be used at an activity of 2×10^{-8} mols of H_2O_2 used/min. The colorimetric method can be standardized against the Balls and Hale method or against the amount of ascorbic acid oxidized (see below) to yield results in terms of absolute units.

Highly purified peroxidase preparations gave some trouble when diluted, due to surface denaturation or a similar phenomenon. This difficulty was not encountered in less pure preparations or more concentrated solutions. This property was utilized to remove peroxidase from ascorbase preparations.

Peroxidase activity may be measured quite easily and accurately by measuring the ascorbic acid oxidized by it when a polyphenol and H_2O_2 are added. This method is rapid and allows expression of activity values in absolute units, such as mols of ascorbic acid oxidized per minute. The measurement is made in the same way as for ascorbase activity except that 2-3 ml. of 0.5 M catechol solution and 1 ml. of 0.1 N H_2O_2 are added to the reaction mixture. Guaiacol may be used instead of catechol. This method also can be used to get initial activity rates covering a period as short as about 10 seconds if desired.

Polyphenolase has heretofore been measured usually by the manometric method, but difficulty has been encountered because of the rapid inactivation of this enzyme.

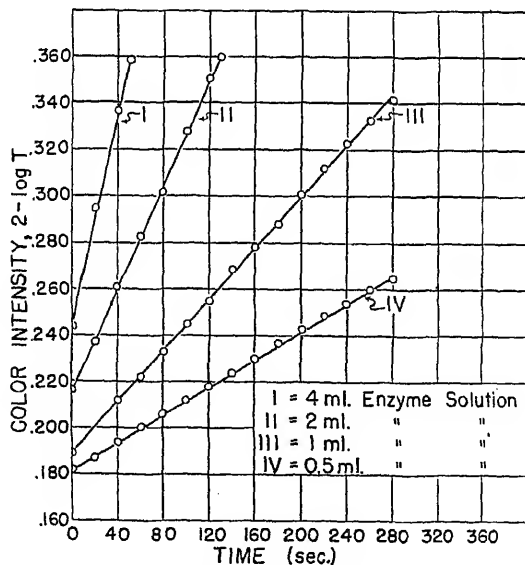


Fig. 1. Measurement of polyphenolase activity, substrate catechol.

Miller and Dawson (26) showed the errors to be as high as 30%. They suggested a method in which the time required to oxidize a definite amount of ascorbic acid by polyphenolase plus added catechol is measured. This is a rather cumbersome method using a 3-necked reaction flask and a continuous siphon to test the reaction mixture from time to time. When all the ascorbic acid is oxidized, the quinone formed is detected by a starch-iodide mixture.

In an attempt to simplify this procedure, the colorimetric method used successfully for peroxidase was applied to polyphenolase and appears to be equally satisfactory for that enzyme. Catechol and pyrogallol are oxidized by polyphenolase and a straight line is obtained when $2\text{-log } T$ is plotted against time. There is very little or no lag period with polyphenolase. Fig. 1 shows that the slopes of the lines are proportional to the enzyme concentration.

The measurements are made in the same manner as for peroxidase, that is, 20 ml. of 0.01 *M* acetate buffer at pH 5.0 and 25°C. is put in an Evelyn colorimeter tube, 2 ml. of 0.5 *M* catechol is added, and 1 ml. of enzyme solution, or more enzyme solution and correspondingly less buffer. The tube is corked, shaken, and placed in the colorimeter. Readings are taken at definite time intervals, using a 420 *mμ* filter, and the values of $2\text{-log } T$ are obtained from the Evelyn chart and plotted against time. Activity is expressed in $\log T$ units/min.

This method compares favorably in precision with the best published methods, having a reproducibility well within 5%, and it is much simpler, requiring only about 5 mins. for an assay (see Fig. 1).

As in the case of ascorbase and peroxidase, polyphenolase activity may also be measured simply and accurately by measuring the amount of ascorbic acid oxidized by the enzyme, under the catalytic influence of added catechol in this case. This method has recently been used and recommended by Sreerangachar (27, 28, 29) in his studies on polyphenolase of tea leaves. He used a reaction time of 1 hr. at 27°C. and pH 5.0–5.4. The above conditions are quite different from those used in this study, and the fact that good results were obtained by both methods indicates that the general method is applicable over a wide range of conditions. However, purified polyphenolase in dilute solution is very easily inactivated and it seems desirable to use as short a reaction time as possible. Also, the temperature should be lower than 27°C. if inactivation is to be avoided.

EFFECTS OF ENVIRONMENTAL FACTORS

The following factors were studied as to their effect on ascorbic acid oxidation by the 3 enzymes: temperature, pH, kind of phenol added, concentration of phenol, hydrogen peroxide concentration (for peroxidase), ascorbic acid concentration, and inhibitors.

Temperatures were varied from 5° to 65°C. with polyphenolase, from 10° to 60°C. with ascorbase, and from 10° to 80°C. with peroxidase. The temperature maxima were respectively 40°, 43° and 60°C. At high temperatures, where autoxidation of ascorbic acid was not

negligible, blanks were run with all reactants except enzyme. Results are shown in Fig. 2.

Both ascorbase and polyphenolase are quite sensitive to heat, polyphenolase especially so. Ludwig and Nelson (30), in a study of the inactivation of polyphenolase during the oxidation of catechol, came to the conclusion that the inactivation involved a definite oxygen uptake per unit of enzyme, which was independent of rate of catechol oxidation and pH, between pH 6 and 7.5. However, they did find that it was dependent on temperature, being lower at 25° than at 35°C., but did

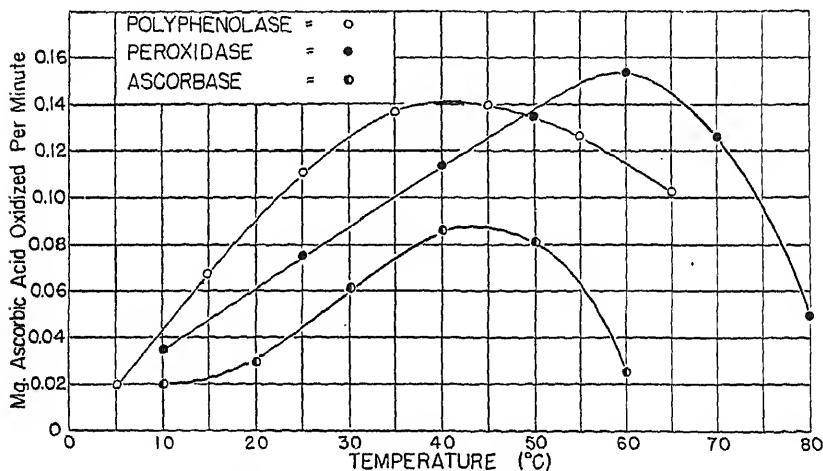


FIG. 2. Effect of temperature on ascorbic acid oxidation.

not try lower temperatures. At lower temperatures the inactivation is not proportional to oxygen uptake; activity curves which decreased in rate in one minute at 25°C. did not decrease for several minutes at 0° to 5°C., although the amount of catechol oxidized was greater than at 25°C.

The effect of pH on ascorbic acid oxidation was measured in a 0.1 *M* oxalate—0.02 *M* phosphate buffer, pH being varied by addition of 6 *N* acid or base. For polyphenolase the effect of pH was also measured in a citrate-phosphate buffer. Autoxidation of ascorbic acid was negligible in the oxalate-phosphate buffer even at pH 9.5 in 8 mins. at 25°C., the time and temperature employed. The effect of pH is shown in Fig. 3. The pH activity curve of polyphenolase is somewhat peculiar in both buffers, since no definite optimum pH value is apparent and the activity is maintained at high pH values. However, this is in line with the stability of the enzyme in highly alkaline solutions.

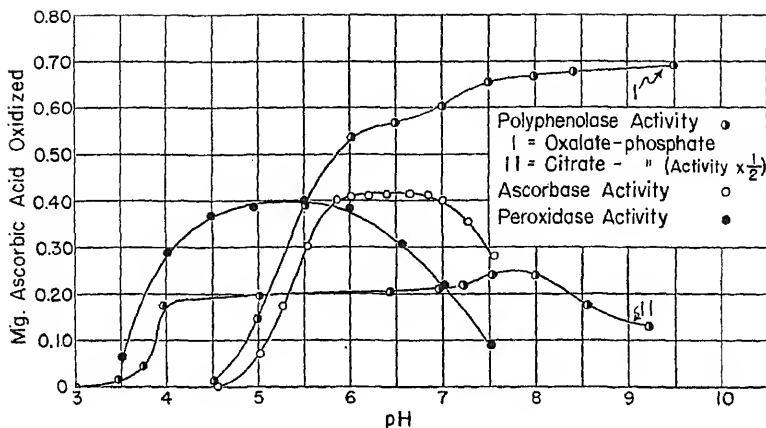


FIG. 3. Effect of pH on ascorbic acid oxidation.

The effect of pH on rate of catechol oxidation by polyphenolase and rate of guaiacol oxidation by peroxidase + peroxide was also determined. In this case both enzymes had definite pH optima, that for peroxidase being at pH 5.7 and for polyphenolase at pH 7.0.

The effect of kind of phenol added was measured in oxalate-phosphate buffer at pH 6 for 3 mins. at 25°C. The concentration of the phenols in the reaction mixture was 0.0057 *M* for polyphenolase and 0.0054 *M* for peroxidase. Of the phenolic compounds tested (catechol, resorcinol, *p*-cresol, phenol, and guaiacol), only catechol was used at all by polyphenolase in the oxidation of ascorbic acid. Peroxidase could use all the compounds to a slight extent but only catechol and guaiacol caused rapid oxidation. The rate of oxidation with guaiacol was about 1.5 times that with catechol.

With pyrogallol, hydroquinone, and tyrosine, the rate of color formation was substituted for ascorbic acid oxidation because of the bleaching effect of the first two on the 2,6-dichlorobenzenone-indophenol dye. Catechol and guaiacol were also used for comparison. Pyrogallol was oxidized by polyphenolase at approximately half the rate of catechol oxidation, while neither hydroquinone nor tyrosine was oxidized at all. Peroxidase with peroxide oxidized pyrogallol at one-seventh the rate of catechol and about one-thirty-fifth the rate of guaiacol oxidation, and also oxidized hydroquinone slightly; tyrosine was not tested.

The effect of catechol concentration on ascorbic acid oxidation was measured for polyphenolase and the effects of both catechol and guaiacol concentrations for peroxidase. It was found that the optimum catechol concentration was 0.05–0.1 *M* for both polyphenolase and peroxidase, but the oxidation rate increased with guaiacol concentration up to the limit of its solubility in water (about 0.1 *M*).

The effect of hydrogen peroxide concentration on ascorbic acid oxidation by peroxidase was measured with catechol as carrier for 3 mins. at 25°C. and pH 6.0. The H_2O_2 concentration in the reaction mixture was varied from 3.8×10^{-5} *N* to 3.8×10^{-2} *N*. The results showed a wide optimum between 0.002 *N* and 0.02 *N* with the highest activity at about 0.006 *N*.

Ascorbic acid concentration was varied from $0.5 \times 10^{-4} M$ to 5×10^{-4} with ascorbase and from approximately 0.7×10^{-4} to $3.7 \times 10^{-4} M$ with polyphenolase and peroxidase. At the lower ascorbic acid concentrations the reaction time was decreased to 1 min. so that not more than about 40% of the ascorbic acid would be oxidized; otherwise a reaction time of 2 mins. was used at pH 6 and $25^\circ C$. The rate of ascorbic acid oxidation was independent of ascorbic acid concentration only above $2-3 \times 10^{-4} M$; below this concentration the rate was apparently directly proportional to ascorbic acid concentration. For peroxidase this applies only to oxidation with catechol as intermediate—with guaiacol the rate was linear down to about $1.3 \times 10^{-5} M$ (95% of added ascorbic acid oxidized).

The inhibitive effects of the following compounds on ascorbic acid oxidation by the 3 enzymes was studied: cysteine-HCl, thiourea, sodium bisulfite, sodium diethyldithiocarbamate, dehydroascorbic acid, and hydrogen peroxide. The added compounds were present in a concentration of $10^{-3} M$, except that sodium diethyldithiocarbamate was added at 10^{-3} , 10^{-4} , and $10^{-5} M$, and hydrogen peroxide at 10^{-2} , 10^{-3} , and $10^{-4} N$. The dehydroascorbic acid was prepared by oxidizing ascorbic acid by filtration through charcoal.

The most effective inhibitor for the copper-containing ascorbase and polyphenolase was sodium diethyldithiocarbamate, which inhibited both 100% at $10^{-3} M$, and inhibited ascorbase activity 98% at $10^{-4} M$ and 23% at $10^{-5} M$. Peroxidase activity was only decreased 19% at $10^{-3} M$, and this was not due to a permanent inhibition but merely a lag period caused by oxidation of the compound. After it was oxidized (in a few minutes), the rate of ascorbic acid oxidation was the same as without added diethyldithiocarbamate.

Hydrogen peroxide was a fairly effective inhibitor of ascorbase activity, causing 98% inhibition at $10^{-2} N$, 55% at $10^{-3} N$, and 0 at $10^{-4} N$. It did not inhibit polyphenolase appreciably. The other compounds tested were less effective, causing 0-75% inhibition. Dehydroascorbic acid caused only 6% inhibition of ascorbase and none of polyphenolase and peroxidase at $10^{-3} M$.

EXPERIMENTS WITH APPLE TISSUE AND EXTRACTS

Fifty g. of fresh apple tissue (Yellow Newton Pippin stored 5 months) was extracted with 150 ml. of ice water for 2 mins. in a Waring Blendor, then filtered through coarse paper. The enzymes present were precipitated by adding 2 volumes of acetone pre-cooled to $-23^\circ C$. After standing 10 mins. the enzyme precipitate was filtered out, dissolved in 50 ml. of ice water, and reprecipitated with 2 volumes of cold acetone. The precipitate was filtered out after 10 mins. and suspended in 25 ml. of ice water, yielding a very turbid, pale pinkish-brown suspension. The natural phenol had all been removed, but polyphenolase and peroxidase were both present, as shown by the following tests:

	Rate of color formation, log T units/min.
1 ml. suspension + 10 ml. pH 7 buffer	0.000
1 ml. suspension + 10 ml. pH 7 buffer + dry catechol	ca. 2.70
1 ml. suspension + 10 ml. pH 7 buffer + 1 ml. 0.04 M guaiacol + 1 ml. 0.1 N H_2O_2	0.500

This suspension was used to test ascorbase activity and polyphenolase activity by measuring ascorbic acid oxidation without and with added catechol, respectively. One ml. of suspension was added to 10 ml. of pH 6.0 oxalate-phosphate buffer containing ascorbic acid and, in the test for polyphenolase activity, also a little dry catechol. The following results were obtained:

	Residual ascorbic acid mg.
Before adding enzyme suspension	0.86
After 3 mins. oxidation by enzyme suspension, no catechol added	0.86
After 1.5 mins. oxidation by enzyme suspension plus added catechol	0.00

Both ascorbic acid oxidation and the rate of darkening in the absence of added ascorbic acid were measured in apple juice under several conditions to provide evidence as to whether polyphenolase or peroxidase was mostly responsible for the oxidations. Balls and Hale (30) stated that darkening of apple juice was due to the action of peroxidase, the peroxide being provided by the action of a direct oxidase, while Wachholder (12) reported peroxidase to be absent in apples. Dawson and Ludwig (31) found that hydrogen peroxide was not formed in the oxidation of catechol by polyphenolase.

To determine whether peroxidase can use peroxide possibly formed by the action of polyphenolase the following experiment was performed: The rates of ascorbic acid oxidation and darkening of (1) polyphenolase plus catechol, (2) peroxidase plus H_2O_2 plus catechol and (3) polyphenolase plus peroxidase plus catechol, were measured in buffered solution, using both the purified enzymes and the crude enzyme suspension free from polyphenols which was used to test for ascorbase activity. The crude enzyme suspension was only employed as a source of polyphenolase, purified peroxidase being added as before, even though the suspension already contained some peroxidase. The procedure was as follows: For ascorbic acid oxidation 10 ml. of pH 6.0 oxalate-phosphate buffer containing about 0.8 mg. of ascorbic acid, and 3 ml. of 0.5 *M* catechol were pipetted into the reaction flask. Then polyphenolase solution, or peroxidase solution plus 1 ml. of 0.2 *N* H_2O_2 , or polyphenolase plus peroxidase was added (the enzyme solutions by rapid-delivery calibrated pipette) and the oxidation was allowed to proceed for 2 mins. When both polyphenolase and peroxidase were used the peroxidase was added first so that no reaction would occur until both enzymes were present. Darkening rates were measured in a similar mixture, except that a pH 5 acetate buffer was employed because the oxalate-phosphate buffer rather strongly inhibited the oxidation of catechol by polyphenolase.

The measurements of ascorbic acid oxidation and darkening were repeated with boiled apple juice as substrate instead of catechol, using purified enzymes. Also ascorbic acid oxidation by purified enzymes was measured in boiled apple juice adjusted to pH 6.0 with NaOH. The results of the above experiments are summarized in Table I.

The results listed in Table I show that peroxidase can promote no detectable oxidation by using peroxide formed by a polyphenolase-catalyzed oxidation of either catechol or natural phenol. The natural phenolic substrate in apple juice is much more rapidly oxidized by peroxidase than by polyphenolase if sufficient H_2O_2 is present, as

TABLE I
*Ascorbic Acid Oxidation and Darkening in Buffer Solutions and Boiled
 Apple Juice by Polyphenolase and Peroxidase*

	Ascorbic acid ox- idized in 2 min.	Darkening rate			
		mg. Purified enzyme	log T units/min. Purified enzyme		Crude enzyme
			I	II	
In buffer solution:					
Polyphenolase+catechol	0.55	0.062	0.029	0.136	
Peroxidase+H ₂ O ₂ +catechol	0.39	0.600	0.600	0.600	
Polyphenolase+peroxidase+catechol	0.58	0.060	0.028	0.136	
Peroxidase+catechol, no H ₂ O ₂ added		0.000	0.000	0.000	
Polyphenolase+catechol+H ₂ O ₂		0.064	0.028	—	
In boiled apple juice (pH 3.75):					
Polyphenolase	0.08	0.045			
Peroxidase+H ₂ O ₂	0.72	0.500			
Polyphenolase+peroxidase	0.07	0.047			
In boiled apple juice adjusted to pH 6:					
Polyphenolase	0.05				
Peroxidase+H ₂ O ₂	0.80				
Polyphenolase+peroxidase	0.04				
Polyphenolase+catechol	2.42				

shown by the results with boiled apple juice. A high concentration of polyphenolase must be added to obtain even a small amount of oxidation, indicated by the rapid rate of oxidation of ascorbic acid when catechol was added.

Since peroxidase of much greater oxidizing power than that of polyphenolase cannot cause an increase in the rate of oxidation over that by polyphenolase alone, when H₂O₂ is not added, it is of interest to discover whether peroxidase has any effect on ascorbic acid oxidation or darkening of fresh juice. Several experiments were conducted with fresh apple juice in order to arrive at a conclusion from different approaches. However, owing to the rapid changes taking place in fresh juice, which contains complete oxidizing systems, the results are rather inconsistent and can only be used to indicate the general trend. The results are summarized in Table II.

TABLE II
Ascorbic Acid Oxidation and Darkening in Fresh Apple Juice

Experiment	Ascorbic acid oxidized in 3 min.		Darkening rate		
	(I)	mg. (II)	$\log T$ units/min. (I)	(II)	
1. Buffer + catechol + juice diluted 180:1 + H ₂ O	0.38	0.24	0.212	0.078	
Buffer + catechol + juice + H ₂ O ₂	0.45	0.26	0.244	0.088	
Buffer + catechol + juice + catalase	0.35	0.24	0.180	0.075	
2. Juice diluted 10:1 + H ₂ O	0.23	0.35	0.072	0.018	
Juice diluted 10:1 + H ₂ O ₂	0.37	0.35	0.112	0.024	
Juice diluted 10:1 + catalase	0.23	0.20	0.040	0.015	
Juice diluted 10:1 + sodium diethyldithiocarbamate, $2 \times 10^{-4} M$			0.000		
			Minutes Standing		
			0-2	6-8	12-14
			0.100	0.055	0.032
			0.157		
0.030					
3. Juice diluted 10:1 + H ₂ O, measured immediately after extraction					
Juice diluted 10:1 + H ₂ O ₂ , measured immediately after extraction					
4. Juice diluted 10:1 + H ₂ O ₂ + catechol					
Juice diluted 10:1 + H ₂ O ₂ + guaiacol					

In the first experiment darkening and ascorbic acid oxidation were measured in buffer solutions with catechol added to eliminate the wide discrepancy in rates of oxidation of the natural phenol by polyphenolase and peroxidase. One ml. of fresh juice diluted 10:1 was added to 15 ml. of reaction mixture, and 1 ml. of either water, 0.2 *N* H₂O₂ or catalase preparation (glycerine extract of horse liver) was added. The rates were measured in the presence of added H₂O₂ to obtain the maximum effect of peroxidase, and in the presence of catalase to eliminate the effect of peroxidase.

According to Balls and Hale (30), fresh apple juice contains hydrogen peroxide, which diminishes as the juice stands. If such is actually the case, it would help explain the difficulty in obtaining consistent results, as the extractions are not always performed in the same length of time. For instance, in Exp. 1 the effect of catalase is measurable in one case and not the other. However, it appears from this experiment that the ratio of amount of peroxidase to amount of polyphenolase in the fresh juice is not over 0.3, based upon the ability of the enzymes to oxidize ascorbic acid or catechol. The actual oxidizing activity of peroxidase is undoubtedly much less than this because of the low concentration of H₂O₂ present, as shown by the values obtained with added catalase.

In Exp. 2 the oxidations were followed in a similar manner in the complete natural system, without added catechol or buffer. Nine ml. of fresh juice diluted 10:1 were used, plus 1 ml. of water, H_2O_2 or catalase preparation; the juice in this experiment could be more concentrated because of the small amount of natural phenol present and the consequently slower oxidation. The results are similar to those in Exp. 1, although in this case the natural phenol present is oxidized about 10 times as fast by peroxidase as by polyphenolase of the same strength. The maximum effect of peroxidase (in the presence of added H_2O_2) may amount to more than 50% of the total in this case, but without added H_2O_2 it is again much less than this.

In Exp. 3 the darkening rates of fresh juice, with and without added H_2O_2 , were measured at different lengths of time after extraction of the juice to show the change in relative activities of polyphenolase and peroxidase as the juice stands. The result indicates that, although peroxidase activity may be high in very fresh juice, it decreases very rapidly and in a few minutes reaches zero or nearly zero. Between 6 and 8 minutes after extraction of the juice the rate with added H_2O_2 was less than without it, and this lower rate was maintained thenceforth; at any given time after the first few minutes the amount of darkening is, therefore, about the same with or without added peroxide. Since a relatively large amount of H_2O_2 was added (1 ml. of 0.2 *N*) the decrease in rate of peroxidase darkening cannot be due to exhaustion of H_2O_2 . It may be due either to inactivation of peroxidase or diminution of phenol. The latter is more probable because, since the rate of darkening of the phenol by peroxidase is much faster than by polyphenolase, a decrease in phenol concentration would have more effect on the peroxidase rate in this system, where both the phenol and peroxidase concentration are low.

In Exp. 4 fresh juice was used only as a source of enzymes, and preferential substrates were used to partially separate the polyphenolase and peroxidase oxidations. Nine ml. of fresh juice was mixed with 1 ml. of 0.2 *N* H_2O_2 and 1 ml. of either 0.1 *M* catechol or 0.1 *M* guaiacol, and the initial darkening rates were measured. The difference in rates can be used as an indication of the relative rates of oxidation by peroxidase and polyphenolase under conditions for maximum peroxidase oxidation, assuming that the fresh juice contained no monophenol oxidase capable of oxidizing guaiacol; there were no indications in this study that such an enzyme was present, and the purified polyphenolase did not oxidize guaiacol at all.

With catechol the total oxidation by peroxidase plus polyphenolase is measured, and with guaiacol only the peroxidase oxidation. It was found (see Effects of Environmental Factors) that peroxidase plus H_2O_2 will oxidize guaiacol approximately 1.5 times as fast as catechol. Therefore, the rate of 0.030 log *T* units/min. with guaiacol would equal 0.020 units/min. when converted to rate of catechol oxidation. Subtracting this value from the value of 0.157 units/min. obtained with catechol leaves 0.137 units/min. as the darkening rate due to polyphenolase. Thus, the minimum ratio of polyphenolase to peroxidase activity under conditions for maximum peroxidase activity (H_2O_2 not limiting) is 0.137/0.020 or almost 7:1.

Under the conditions encountered with reasonably freshly harvested apples, peroxidase would probably have little effect even initially upon the darkening rate, because there is a considerable amount of ascorbic

acid present in fresh apples which would be oxidized and thus consume any available H_2O_2 before darkening could start. The apples used in the above experiments were very low in ascorbic acid owing to long storage.

In the above experiments darkening and ascorbic acid oxidation are seen to correlate very well. This would be expected on the basis of the generally accepted theory of ascorbic acid oxidation by polyphenolase and peroxidase, in which a phenol is oxidized by the enzyme and in turn oxidizes ascorbic acid. As long as any ascorbic acid remains, the phenol acts only as an intermediate and no color appears. After the ascorbic acid is all oxidized the phenol becomes oxidized and colors the reaction mixture. That this is true for solutions has been stated by Samisch and Cruess (32), Miller and Dawson (33), Hussein and Cruess (24), and Sreerangachar (34) and confirmed by the authors. Joslyn *et al.* (35) found a correlation between darkening in artichokes, which contain polyphenolase, and ascorbic acid oxidation. Applying this theory to fruits such as apples, no darkening of a cut surface or of juice should occur until all the ascorbic acid is oxidized. This evidently is actually the case, although the period elapsing from the time the apple tissue is exposed to air until darkening begins is extremely short. In the foregoing experiments, initial ascorbic acid was measured by adding juice to the reaction vessel containing ascorbic acid, then adding strong oxalic acid immediately. This operation took only 4 or 5 secs., but it was found that the ascorbic acid value was zero when 9 ml. of fresh apple juice was added to 16 ml. of reaction mixture and tested in this manner. To obtain a satisfactory initial value of ascorbic acid, it was necessary to dilute the juice 10:1 with water and use about 1 ml. of this in 16 ml. of reaction mixture.

This extremely rapid rate of oxidation should cause the loss of 20 mg. of ascorbic acid in a 100 g. apple in 12 secs. if the apple were ground and exposed to air. Such a rate of oxidation has been roughly confirmed experimentally—no ascorbic acid could be found in apples which were ground and immediately dropped in oxalic acid, in less than 30 secs. However, if the same apples were quartered into acid and then blended, they had up to 20 mg. ascorbic acid/100 g. The failure to prevent this enzymic oxidation together with autoxidation has been a major cause of the wide variation in ascorbic acid values reported for the same plant material in the earlier literature; in fact, most of the values reported prior to about 1942 are decidedly untrustworthy. Citrus juices are an

exception, because in these there is only a small enzymic and a small autoxidation of ascorbic acid.

SUMMARY

1. New methods or modifications were developed for measuring the activities of ascorbase, peroxidase and polyphenolase. For ascorbase the measurement is made in oxalate-phosphate buffer of pH 6.0, in which non-enzymic oxidation is negligible. A known amount of ascorbic acid is added and, after a short time of reaction, the residual ascorbic acid is measured colorimetrically. For peroxidase, the rate of color formation when guaiacol is oxidized by H_2O_2 under its catalytic influence is used as a measure of activity. The rate of color formation is linear for several minutes in the pH 5.0 acetate buffer employed. The slope of the rate curve is proportional to enzyme activity. For polyphenolase a similar method is used, except that catechol is oxidized instead of guaiacol, and no H_2O_2 is added.

Peroxidase and polyphenolase were also measured by their rate of ascorbic acid oxidation. In this method, guaiacol or catechol plus H_2O_2 is added in the peroxidase measurement, and catechol alone is added in the polyphenolase measurement.

2. Ascorbase, peroxidase and polyphenolase were purified to remove phenolic compounds and to separate the enzymes one from the other. The purifications mainly involved cold acetone precipitation after extraction in a blender. Peroxidase was removed from the other enzymes by several filtrations, as it is very sensitive to surface denaturation. Salts were removed from polyphenolase preparations by the use of synthetic resin ion exchangers instead of by dialysis, because dialysis caused much inactivation.

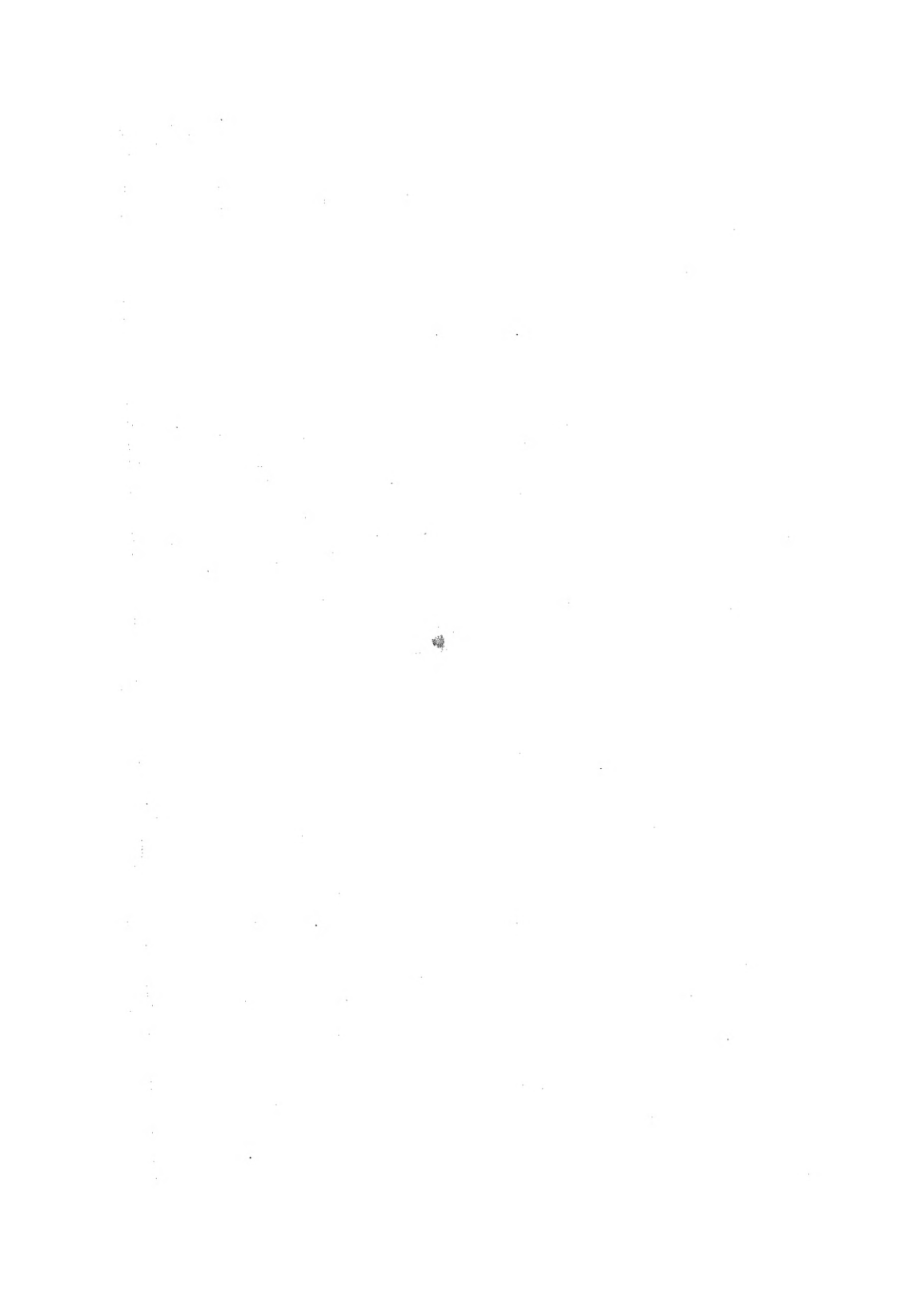
3. The effect of temperature, pH and other factors on the kinetics of oxidation of ascorbic acid by ascorbase, peroxidase and polyphenolase was studied.

4. The mechanism of ascorbic acid oxidation and darkening in apples was investigated in an attempt to ascertain which enzyme or enzymes were mostly responsible for the oxidation. Apples were selected as being more or less typical of fruits in the oxidizing enzyme systems they contain. It was found that: (1) ascorbase is absent in apples; (2) ascorbic acid oxidation is intimately related to darkening, the latter not occurring until all the ascorbic acid is oxidized, although this may

take only a few seconds; (3) polyphenolase is largely responsible for both ascorbic acid oxidation and darkening, peroxidase oxidation being of little importance.

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Some Observations on the Isolation of Adenosine Triphosphate from Skeletal Muscle

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INTRODUCTION

The following is an account of investigations on the isolation of adenosine triphosphate (ATP) from the skeletal muscle of the rabbit, sheep, and turkey.

Rabbit muscle has been the most commonly used source of ATP and in most of the methods fresh muscle tissue has been employed (1, 2, 3, 4, 5, 6). Lohmann and Schuster (7) have pointed out that delay in the working up of muscle after death of the animal causes extensive decomposition of ATP, due to rapid *post mortem* changes, and that the final product obtained from such tissue contains very little ATP. Several workers have reported the preparation of ATP from the skeletal muscle of other species. Koenig and Svarz (8) reported the preparation of ATP from beef muscle obtained from the slaughter floor as soon as possible after the death of the animal. The yield of the anhydrous barium salt was 1.2–1.3 g./kg. of muscle. Barrenscheen and Filz (9) obtained ATP from the fresh muscle of dogs, sacrificed by shooting. The yields varied within considerable limits, namely, 0.2–0.4 g. of the silver salt/100 g. of muscle. Szent-Györgyi (10) described the preparation of dry muscle powder of horse and rabbit, obtained by desiccation with alcohol, as a stable and convenient source of ATP. In the case of both rabbit and horse muscle powder the yield of ATP corresponded to about 1.25 g. of free ATP/kg. of fresh muscle.

Our investigations were primarily concerned with finding suitable and stable sources of ATP that could be used as starting material for preparations assigned as a laboratory exercise for graduate students in biochemistry. The providing of fresh rabbit muscle to several groups of students, so that the latter could complete the 2 extractions with trichloroacetic acid and the first heavy metal precipitation during the same laboratory period (2–3 hrs.), is a heavy strain on the instructing staff, and we, therefore, undertook investigating the possibility of

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storing muscle tissue in some suitable manner without undue losses in yield of ATP and without detracting appreciably from the purity of the final product. In addition, we attempted to shorten the time of preparation by using the frozen powdered whole rabbit carcass as a source of ATP. Mention may be made in this connection that Lutwak-Mann (11) reported the presence of acid-soluble phosphorus in rabbit bone marrow, of which more than 25% was present in a form hydrolyzable by *N* hydrochloric acid in 7 mins. In our preparations, using turkey muscle, we separated the white muscle from the red muscle. An examination of the analytical figures reported by Lohmann (12) shows that the pyrophosphate content of trichloroacetic acid extracts of white muscle of rabbit is nearly 1.5 times that of the red muscle. We selected the turkey as a suitable source of white and red muscle, inasmuch as the two types of muscle occur quite distinct and large quantities of each can be obtained from the same animal for the preparation of ATP.

EXPERIMENTAL

Preparation of Sample

Rabbits were usually anesthetized with magnesium sulfate as recommended by Dubois, Albaum and Potter (13). A few experiments were also carried out using nembutal as anesthetic. The lamb used in the experiment was anesthetized by intrajugular injection of 200 mg. of chloral hydrate/kg.² The turkey was anesthetized with nembutal. The animals were bled to death by cutting the carotid arteries, decapitated, skinned, eviscerated and any gross fat deposits cut out. These operations took about 5–10 mins. in the case of the rabbit, and about 30 mins. each in the case of the lamb and turkey. The dressed carcasses were chilled in ice water or crushed ice and maintained for about an hour in the cold room (5°C.).

In the experiments with whole rabbit, the carcass was cut into half a dozen parts and frozen by packing with dry ice. The material was then chipped into smaller bits with heavy butcher's cleavers and powdered in a laboratory mill equipped with grinding plates,³ dry ice being intermittently fed to the machine to prevent the carcass from thawing. This procedure gave a very satisfactory powdered product.

Extraction with Trichloroacetic Acid and Precipitation of ATP

About 1–1.2 kg. of muscle tissue were used in each experiment. The method of extraction and subsequent precipitation of ATP was essentially that of Dounce and co-workers (6). We have, however, considered it advisable to drop the muscle bits,

² The anesthesia and bleeding of the lamb were kindly carried out by Dr. H. H. Dukes in the Physiology Department.

³ Model 4E Quaker City Mill, Arthur H. Thomas Co., Philadelphia.

as soon as they are cut, directly into weighed ice cold trichloroacetic acid, and to blenderise as soon as 200 g. of the material had accumulated. This technique of dropping the muscle immediately into ice cold trichloroacetic acid without waiting to work up the whole carcass, is definitely advantageous over the usual method of removing all the muscle tissue and finally extracting with trichloroacetic acid. In this way, the trimming of the muscle free from fat and connective tissue can be carried out more thoroughly and more time is available for cutting out the last major bits of muscle from the carcass, without risking any considerable enzymatic breakdown of ATP. With muscle tissue, the grindings and filtration were carried out in the cold room. As already mentioned, the white and red muscles of turkey were separately processed. Due to the peculiar consistency of the white muscle we have used 2.5 volumes of trichloroacetic acid, both for the first and the second extractions. Powdered whole frozen rabbit carcass containing excess "dry ice" was dropped into trichloroacetic acid at room temperature with vigorous stirring and, when most of the carbon dioxide had evolved, the pasty material was ground in the Waring Blender at room temperature.

To study the effect of storage of muscle in the frozen condition, 3 sets of experiments were set up with rabbit muscle. In one, the muscle tissue, without addition of any trichloroacetic acid, was allowed to freeze slowly by keeping in the freezing chamber at -17.8°C . and stored in the same place for a week. The frozen mass was allowed to thaw at room temperature and then extracted with trichloroacetic acid. In the second set of experiments, the muscle was frozen as above, without any trichloroacetic acid, but the frozen mass was not allowed to thaw out before addition of trichloroacetic acid; the material was covered with trichloroacetic acid at room temperature and, when the outside tissue had become sufficiently soft to permit the tipping of the material, it was transferred to the blender and ground at room temperature. In the third series of experiments, the muscle was frozen in contact with 2 volumes of trichloroacetic acid. In the case of sheep and turkey muscle, freezing in contact with 2 volumes of trichloroacetic acid only was carried out.

To follow quantitatively any changes during storage in the amount of hydrolyzable phosphorus, we have carried out estimations of pyrophosphate on aliquots of the trichloroacetic acid extracts, according to the method of Lohmann (14). The latter figures were obtained as the difference between the value of orthophosphate obtained after 7 minutes' hydrolysis with *N* hydrochloric acid at 100°C . and the directly estimated total inorganic phosphorus of the extract (that is, the sum of "true" inorganic phosphorus and phosphocreatine phosphorus).

The final barium salt was collected in centrifuge tubes, dried in a vacuum desiccator over calcium chloride or Drierite for 24-48 hrs., powdered, and weighed.

For analyses, a portion of the dried product was weighed out into a mortar, and the solid ground with water to which dilute sulfuric acid in slight excess of the calculated amount was added. The precipitated barium sulfate was centrifuged off and repeatedly washed by grinding with water faintly acidulated with sulfuric acid and separating on the centrifuge. The supernatants were combined and made up to volume. Aliquots of this solution were used for estimation of total phosphorus, inorganic phosphorus and phosphorus hydrolyzable in 7 and 15 mins. by heating with *N* hydrochloric acid at 100°C . Our results are summarized in Table I. All the phosphorus estimations were carried out by the method of Sumner (15), using ferrous

TABLE I

Yield and Analysis of Adenosine Triphosphate Prepared from the Skeletal Muscle of Different Species

Nature of sample	Hydro- lyzable P in TCAA ^a extract	Yield of ATP	Phosphorus analysis of ATP			
			Total	Inor- ganic	Hydrolyzable (N HCl at 100°C.)	
					7 min.	15 min.
I. Theoretical for Ba ₂ ATP·4H ₂ O	mg./kg.	mg./kg.	per cent	per cent of total P	per cent of total P	
			10.94	nil	66.7	
II. Rabbit muscle						
(1) Fresh						
(a) Nembutal anesthesia	285	2800	10.96	1.0	65.4	68.6
(b) MgSO ₄ anesthesia	331	3400	10.96	1.2	64.1	67.1
(2) Frozen and thawed	35	30	9.52	7.6	74.4	
(3) Frozen, mixed with TCAA before thawing	137.5	710	10.17	2.8	61.6	65.3
(4) Frozen with TCAA	240	3100	11.08	1.6	63.1	69.1
III. Whole rabbit carcass	323	1200	9.75	6.8	61.8	
IV. Lamb muscle						
(1) Fresh	408	2750	10.58	1.8	62.8	66.8
(2) Frozen with TCAA	53.5	150	10.0	3.1	58.9	62.9
V. Turkey muscle						
(1) White						
(a) Fresh	422	5050	10.91	0.8	65.1	68.2
(b) Frozen with TCAA	307	2250	10.61	1.4	64.0	67.0
(2) Red						
(a) Fresh	126	1300	10.47	1.2	62.3	66.3
(b) Frozen with TCAA	162	300	10.39	2.2	60.8	64.0

^a Trichloroacetic acid.

sulphate as reducing agent. It is to be noted that we have expressed inorganic phosphorus as percentage of total phosphorus and not on per cent basis of the weight of ATP, as is conventionally done. The hydrolyzable phosphorus was corrected for the inorganic phosphorus and expressed as per cent of the corrected total organic phosphorus of the compound. For estimating water content, specimens were dried for

about 3 hrs. in the Abderhalden apparatus over phosphorus pentoxide at a temperature of 100°C., the apparatus being evacuated by a Hyvac pump. About 8% of water was lost in this way, as compared with the theoretical figure of 8.47% calculated on the basis of formula $\text{Ba}_2\text{ATP} \cdot 4\text{H}_2\text{O}$.

Lohmann (2) pointed out that air-dried barium salt of ATP undergoes a certain amount of decomposition on storage. After a few months storage, barium fractionation showed decomposition into adenylic acid and pyrophosphate. We have been storing our products in glass stoppered bottles and keeping them under desiccation in the cold room. We have not run tests by barium fractionation to follow the stability of our preparation, but we reanalyzed for inorganic and hydrolyzable phosphorus a preparation of ATP obtained from fresh rabbit muscle and stored for about 10 months; no detectable variation in either figure was obtained.

DISCUSSION

Storage of rabbit muscle in the frozen condition causes a marked alteration in the amount of hydrolyzable phosphorus (which is a rough indication of the amount of ATP) in the extracts, depending upon the condition of the freezing and the subsequent extraction. We wish to emphasize the fact that we have used the figures for hydrolyzable phosphorus in the trichloroacetic acid extracts only as a roughly quantitative indication of the amount of ATP in the extracts. Several authors have pointed out (*cf.* Stone, 16) that pyrophosphate determinations direct on tissue extracts suffer from the disadvantage that besides pyrophosphate, some other phosphate esters are also partially split, as, for example, hexose phosphates and triose phosphates. In the case of the final barium salts, however, we have depended almost entirely on the ratio of hydrolyzable phosphorus to total organic phosphorus as an indication of the purity of the compound.

For the purpose of the present investigation, namely, a study of the suitability of muscle tissue of different species of animals and a comparative study of the use of fresh and frozen muscle, we have considered it unessential to report a complete analysis of the final product, especially in view of the fact that Dounce and coworkers, whose method of isolation we have closely followed, have reported such an analysis, including, besides total and hydrolyzable phosphorus, also pentose, barium and nitrogen. Different authors have reported different periods for the completion of the splitting of pyrophosphate from ATP by the action of *N* acid at 100°C. Lohmann (1) stated that exactly $\frac{2}{3}$ of the total organic phosphorus was converted into orthophosphate in 7 mins. in the presence of *N* hydrochloric acid at 100°C. On the contrary,

Fiske (17) and Kerr (3) found that $\frac{2}{3}$ of the total phosphorus was split off only in 15 mins. by *N* acid at 100°C. The latter author reported that, for 22 different preparations of the barium salt, each with a P:N ratio close to the theoretical figure, the amount of phosphorus hydrolyzed within 15 mins. averages $64.9 \pm 1.4\%$ of the total. Our own figures show that 62–65% of the total organic phosphorus is split off in 7 minutes' time by *N* hydrochloric acid at 100°C. and 66–68% at the end of 15 mins.

Trichloroacetic acid extracts from fresh muscle of rabbit anesthetized with magnesium sulfate, contain on the average 330 mg. of hydrolyzable phosphorus/kg. muscle; when the muscle is stored frozen for a week and subsequently thawed at room temperature before extraction, the figure for hydrolyzable phosphorus drops to a very low value and the final yield of ATP is practically negligible. Also, analysis of the product shows it to be highly contaminated. This loss of ATP might have occurred during 3 stages—during the period (2–4 hrs.) that elapses before the muscle mass becomes frozen in the freezing room, during the one week period of storage in the frozen condition and during the thawing of the frozen mass. That the third factor can assume considerable proportions is shown by the figures for Expt. No. 3, where the frozen tissue was not allowed to thaw out to any considerable extent, but was ground up with trichloroacetic acid, while still to a large extent in the frozen state. Even in this case the yields were not satisfactory. The best results were obtained when muscle tissue was frozen in contact with trichloroacetic acid. The figures for hydrolyzable phosphorus in the initial extract and the yield of the final product and its purity compare favorably with the figures for fresh muscle.

The yield of ATP obtained by us from lamb muscle is considerably higher than the reported yields from beef and horse muscle. Lamb muscle, however, was found to be extremely sensitive to storage, even in contact with trichloroacetic acid; the extracts from stored muscle contain less than $\frac{1}{4}$ of the hydrolyzable phosphorus of fresh muscle extract. The yield of the final product was only 150 mg./kg. of muscle as compared with 2750 mg. from fresh muscle. Analysis of the product showed it to be far from pure. One possible cause for this loss might have been the inevitable delay (about 2 hrs.) that occurred before the muscle (which was throughout maintained at low temperature) could be cut up and placed in contact with the acid.

The figures for turkey muscle are very interesting. The hydrolyzable phosphorus in the case of fresh white muscle is over 2.5 times that of fresh red muscle. The final yield of ATP from the former is more than 3 times that from the latter. The authors are not aware of any satisfactory explanation for the marked difference in the ATP content of the white and red muscles. It is usual (18) to think of red muscles as slowly contracting tonus muscles, using up small amounts of energy, whereas the white muscles are involved in phasic contractions, which are accompanied with considerable expenditure of energy. The difference in ATP might be related with the difference in function of the 2 muscles. It will be noted that turkey white muscle gives an exceptionally high yield of ATP, namely, 5 g./kg. of fresh muscle. The white breast muscle of turkey would, therefore, form an ideal source for the large scale production of ATP. There is an added advantage in the fact that there is very little fat deposit or fascia attached to the white muscle, so that the muscle can be cut out very rapidly as compared to the red leg muscle which contains large amounts of tendons, fat, *etc.* Neither the white nor the red muscle of turkey is well suited for storage in frozen condition. After 5 days' storage in contact with trichloroacetic acid, the yield of ATP from white muscle is less than half that from fresh muscle, whereas that from stored red muscle is much smaller. Analyses of the trichloroacetic acid extracts show that there has been no decrease in the pyrophosphate; in fact, the red muscle extract shows a marked increase. We are not able to account for these observations at present.

The extracts from whole rabbit carcass contain considerable amounts of hydrolyzable phosphorus namely 323 mg./kg. of carcass. Considering the fact that 1 kg. of carcass would yield about 500–600 g. of muscle tissue under working conditions (that is, rejecting the breast and abdominal muscles, and other thin strips of muscle tissue with heavy coatings of fascia), this figure would indicate that the extract from whole rabbit carcass would contain considerably higher amount of ATP than the extract from the muscle mass that can be obtained under practical conditions from 1 kg. of carcass. But the actual working up of the extract was attended with experimental difficulties which we have thus far been unable to overcome, one of them being the presence of large amounts of tricalcium phosphate. The yields and the purity of the final product were not as satisfactory as might have been anticipated from the analysis of the initial extract.

ACKNOWLEDGMENT

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SUMMARY

1. Rabbit muscle can be stored frozen in contact with trichloroacetic acid for several days without undue losses in the yield of ATP. When the muscle tissue is frozen as such without the addition of trichloroacetic acid, extensive decomposition of ATP takes place; a certain proportion of this decomposition occurs during the process of thawing.

2. Sheep muscle can be conveniently used as a source of ATP when large amounts of the latter are needed. The yield has been about 2.75 g. of the barium salt/kg. of muscle as compared with 3-3.4 g./kg. fresh rabbit muscle. Sheep muscle, however, does not lend itself to storage in the frozen condition for any length of time.

3. The white muscle of turkey appears to be the richest source of ATP. The yield of the purified barium salt was as high as 5 g./kg. of fresh muscle. White muscle contains a much higher amount of ATP than red muscle. Turkey muscle is not as well suited for storage in the frozen state as rabbit muscle.

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The Inhibition by Fluoroacetate and Fluorobutyrate of Fatty Acid and Glucose Oxidation Produced by Kidney Homogenates¹

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INTRODUCTION

It has been shown (1, 2) that fluoroacetate³ inhibits the respiration of tissue slices and of yeast and some bacteria mainly through an inhibition of acetate metabolism. In liver slices, there has also been found inhibition of butyrate, caproate, and valerate oxidation. To study more satisfactorily the influence of this halogen acid on fatty acid metabolism, a number of ground animal tissue suspensions were prepared. Rabbit kidney cortex proved the most satisfactory, since appreciable increases in the oxygen uptake were obtained in the presence of a large number of fatty acids. The experiments presented in this paper show that fluoroacetate as well as fluorobutyrate do not possess, in animal tissues, the specificity that they showed in yeast. Both fluoroacids inhibited equally the oxidation of a number of fatty acids. Furthermore, while fluoroacetate in yeast inhibited the synthesis of citric acid and had no effect on the oxidation of glucose, in kidney homogenates it increased the synthesis of citrate and inhibited the oxidation of glucose. These experiments were performed in 1945.

EXPERIMENTAL

The kidneys were obtained from rabbits immediately after the death of the animal. The cortex was separated, and ground in a dry, cold mortar and in a glass homogenizer.

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³ Fluoroacetate will be designated as FA, fluorobutyrate as FB.

In general, 100 mg. of tissue were used per vessel; the tissue was suspended in 2 cc. Krebs' phosphate-saline buffer, pH 7.4 (0.02 *M* phosphate). The fatty acids were used as the sodium salts. Kidney cortex preparations obtained from rats showed no extra O_2 uptake over the blank values with acetate as substrate. Mouse kidney cortex preparations showed only a slight increase. NaFA and NaFB were prepared from the methyl esters kindly provided by Professor M. S. Kharasch, of the Department of Chemistry of this University. Acetic acid was determined by the method of Friedemann (3), citric acid by the method of Pucher *et al.* (4).

Oxidation of Fatty Acids by Rabbit Kidney Cortex Suspensions

Muñoz and Leloir (5), and Lehninger (6, 7), have shown that suspensions of rat liver and heart oxidize fatty acids readily. Homogenized suspensions of rabbit kidney cortex were able to oxidize a large number of fatty acids as shown by the increase in the O_2 uptake (Table I). Of 15 fatty acids used as substrates, valerate and α -bromo-

TABLE I

Oxidation of Fatty Acids by Rabbit Kidney Cortex Suspensions

Kidney suspension, 1 cc. (130 mg. wet weight in phosphate-NaCl buffer); phosphate-NaCl buffer, pH 7.4, 1 cc.; substrate (0.02 *M*), 1 cc. Temp. 38°C. Gas phase, air. The O_2 uptake in the absence of air has been subtracted.

	O_2 uptake in 1 hr.	
	Fresh sus- pensions	5 hrs. old
	mm. ³	mm. ³
Formic	12	
Acetic	89	
Propionic	44	12
Butyric	176	
α -Hydroxybutyric	17	
β -Hydroxybutyric	138	
α -Ketobutyric	47	0
Crotonic	85	
Vinylacetic	127	
α -Ketovaleric	85	71
Valeric	0	
Methylethyl acetic	132	
α -Bromovaleric	0	
Caproic	220	118
α -Ketocaproic	154	82

valerate were the only ones which showed no effect. However, an isomer of valeric acid, methyl ethyl acetate, was readily oxidized, as was α -ketovalerate. The lack of oxidation of valerate may have been due to the presence of small amounts of impuri-

ties. There was little oxidation of formate or α -hydroxybutyrate, whereas the preparation was quite active on β -hydroxybutyrate. α -Ketobutyrate and propionate showed small but definite increases in oxygen consumption.

The effect of varying amounts of tissue suspension on the rate of oxidation of acetate and butyrate is shown in Table II. With 30 mg. of tissue there was no oxidation of acetate. Oxidation started with 50 mg., 150 mg. being the optimal amount per vessel. The increase in O_2 uptake with butyrate as substrate was about 1.5–2 times as great as with acetate as substrate.

TABLE II
*Effect of Concentration of Kidney Suspensions on the
Oxidation of Acetate and Butyrate*

Substrate	Tissue weight	O_2 uptake in 1 hr.		
		With substrate	No substrate	Increase
Acetate	mg.	mm. ³	mm. ³	per cent
	30	10	10	None
	50	27	18	50
Butyrate	100	192	106	81
	50	30	10	200
	100	218	102	104
	150	378	245	54
	200	625	450	39

Some attempts were made to prepare a cell-free enzyme preparation which would oxidize acetate and butyrate. However, the enzymes for fatty acid oxidation in kidney homogenates were found to be very labile. For example, in one hour, a fresh preparation took up 180 mm.³ O_2 (with no substrate); 268 mm.³ with butyrate; 207 mm.³ with acetate. After 24 hours, the corresponding O_2 uptakes were 52, 62, and 59 mm.³ Four to five hr. suspensions gave only half-values for acetate oxidation, although other substrates were readily oxidized. The enzymes were impaired also by incubation at 37°C. for 40 mins. in the absence of substrate.

Kidney suspensions prepared from rabbits 5 hrs. after the animal died (kidneys kept at 3°C.) had only half the normal activity. Freezing the kidney overnight in a dry-ice cabinet produced complete loss of acetate and butyrate oxidation. The fatty acid oxidation enzymes in these preparations seemed associated with the solid particles. Washing the preparation once resulted in loss of half its activity; freezing, or freezing and drying, produced complete loss.

*Effect of FA and FB on the Oxidation of
Fatty Acids by Kidney Suspensions*

FA and FB at concentrations of 0.001 *M* produced an immediate, and practically complete, inhibition of acetate oxidation by rabbit kidney cortex (Fig. 1). This strong inhibition of acetate oxidation by FB is in striking contrast with the complete lack of action on the oxidation of acetate by bakers' yeast (2). A reason for this discrepancy might be the possible oxidation of FB into FA.

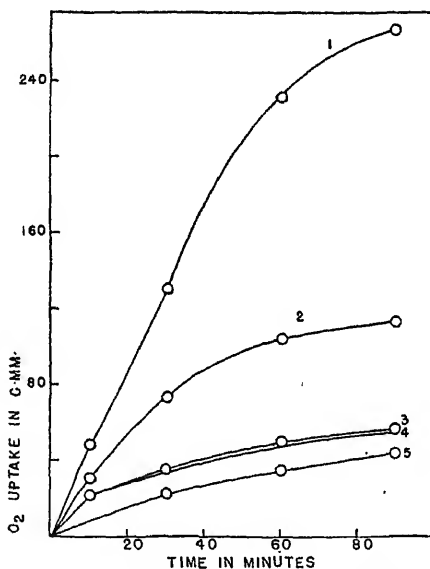


FIG. 1. Effect of FA and FB (0.001 *M*) on the oxidation of acetate by rabbit kidney homogenates. 1. Acetate; 2. No substrate; 3. Acetate + FA; 4. No substrate + FA; 5. Acetate + FB.

To explore this possibility, FA and FB (0.01 *M*) were incubated at 37°C. for 3.5 hrs. with kidney cortex suspensions. The solid matter was centrifuged off and 0.3 cc. of the filtrates from kidney (Filt. 1), kidney plus FA (Filt. 2), and kidney plus FB (Filt. 3), were added to yeast suspensions in phosphate buffer, pH 7, after which acetate was added from the side arm of the Warburg vessels. The final concentration of the fluoroacids was 0.001 *M*. Yeast plus Filt. 1, used in one hour 345 mm.³ O₂; yeast plus Filt. 2, 52 mm.³; yeast plus Filt. 3, 363 mm.³ After 3.5 hrs. of incubation with kidney suspension, FB had no effect at all on the oxidation of acetate by yeast, an indication that the inhibition of acetate oxidation in kidney homogenates was not due to its transformation into FA.

Another striking difference in the action of FA on yeast cells and tissue suspensions was the lack of reversibility of the inhibition in tissue slices. Thus, while in yeast cells the inhibition was released after some time had elapsed (2), in kidney suspensions there was no change

TABLE III

Effect of FB on the Oxidation of Butyrate by Kidney Suspensions

Kidney suspension, 150 mg. per vessel; butyrate, 0.02 M. Total volume, 3 cc. Gas phase, air. Duration of experiment 30 mins.

FB	Inhibition of O ₂ uptake	
	With butyrate	No substrate
<i>M</i>	<i>per cent</i>	<i>per cent</i>
1×10^{-3}	complete	60
3×10^{-4}	90	59
8×10^{-5}	83	29
5×10^{-5}	79	25
2×10^{-5}	41	6
1×10^{-5}	16	5
5×10^{-6}	2.4	3

in inhibition with time. Furthermore, the release of FA inhibition by ethyl alcohol in yeast cells, found by Black and Hutchens (8), did not occur in kidney suspensions, where addition of ethyl alcohol, either before or after addition of FA, had no effect at all on the degree of inhibition of acetate oxidation.

The effects of FA and FB on the oxidation of butyrate were quite similar to the inhibition produced on the oxidation of acetate. Both

TABLE IV

FB and FA Inhibition of Butyrate Oxidation by Kidney Suspensions

Inhibitor concentration	Inhibition butyrate oxidation		No substrate	
	FB	FA	FB	FA
<i>M</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1×10^{-3}	complete	complete	70.3	45
8×10^{-4}	97.8	88.3	59.4	40
4×10^{-4}	97.2	82.4	49.7	39
8×10^{-5}	93.3	41.5	45	12.7
5×10^{-5}	80.2	32	35.5	17

produced an immediate and complete inhibition. FB proved to be a potent inhibitor for butyrate oxidation by rabbit kidney, as 0.00001 *M* produced appreciable inhibition (Table III). Comparison of the degree of inhibition produced by FA and FB showed FB a more effective inhibitor than FA (Table IV). Furthermore, the inhibitions produced by FB were more consistent than those produced by FA, for occasionally kidney cortex suspensions were found resistant to the inhibitory action of FA.

The inhibitory effect of FA and FB was extended to the oxidation of several other fatty acids (Table V). When the concentration of inhibi-

TABLE V

Effect of FA and FB on the Oxidation of Fatty Acids by Kidney Suspensions

Rabbit kidney, 100 mg.; substrate, 0.02 *M*; FA and FB, 0.001 *M*. Volume per vessel, 3 cc. Duration of experiments, 60 mins.

Substrate	Inhibition	
	FA	FB
	<i>per cent</i>	<i>per cent</i>
Acetate	complete	84
Butyrate	96	complete
α -Ketovalerate	complete	complete
Caproate	complete	complete
α -Ketocaproate	complete	93

tors was diminished to 0.00005 *M*, FB inhibited butyrate oxidation 86.5% and caproate oxidation 81%. At the same concentration FA produced inhibitions of 32 and 11%, respectively.

Effect of FA and FB on the Oxidation of Carbohydrate Intermediates

The inhibitory effect of these two halogen acids was not confined to the oxidation of fatty acids. The oxidation of glucose was almost completely inhibited by both FA and FB, although there was no effect at all on the oxidation of citrate, α -ketoglutarate, and malate in experiments of 30 mins. duration (Table VI). The oxidation of lactate by kidney cortex homogenate was appreciably inhibited by both FA and FB. However, lactic acid dehydrogenase prepared from the same tissue was not inhibited when the reaction

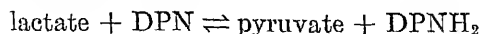


TABLE VI

Effect of FA and FB on the Oxidation of Carbohydrate Intermediates by Kidney Suspensions

Substrates, 0.02 M; FA and FB, 0.001 M. Duration of experiments, 30 mins. The blank O₂ uptake has been subtracted.

Substrates	O ₂ uptake		FB
	Control	FA	
	mm. ³	mm. ³	mm. ³
None	148	92	85
Glucose	168	5	15
Lactate	155	92	64
Citrate	136	139	141
α -Ketoglutarate	147	152	158
Malate	161	183	160
Acetate	116	0	7

was studied spectrophotometrically. A lactate oxidase system obtained from gonococci, where the oxidation of lactate to pyruvate was measured by the O₂ uptake, was also unaffected by both FA and FB. To demonstrate that the oxidation of lactate to pyruvate was not the process inhibited by FA and FB, the experiments were performed in the presence of 0.0001 M *p*-carboxyphenylarsenoxide. This arsenical, at that concentration, inhibits pyruvate oxidation but has no effect on the oxidation of lactate to pyruvate: (9). There was no inhibition by FA or FB (Table VII). It may be concluded, therefore, that the

TABLE VII

Effect of FA and FB on Lactate Oxidation by Kidney Suspensions and by Lactate Oxidase

Experimental	Inhibition	
	FA	FB
	per cent	per cent
Kidney suspension, O ₂ uptake	4	55.6
In the presence of <i>p</i> -carboxyphenylarsenoxide (1×10^{-4} M), O ₂ uptake	None	None
Lactic dehydrogenase extracted from kidney, DPN reduction	None	None
<i>Gonococci</i> (old suspension), O ₂ uptake	None	None

TABLE VIII

*Effect of FA on the Oxidation and Decarboxylation
of OA by Kidney Suspensions*

Tissue, 150 mg.; OA, 0.01 M; FA, 0.01 M; BaCl₂, 0.017 M. Total volume, 3 cc.
Duration of experiments, 1 hr.

Substrate	O ₂ uptake (-) or CO ₂ output (+)		Inhibition per cent
	Control	FA	
No substrate, O ₂ as gas phase	-106	- 46	56
OA, O ₂ as gas phase	-206	-237	None
No substrate, N ₂ :CO ₂ as gas phase	+ 5	+ 3	None
OA, N ₂ :CO ₂ as gas phase	+151	+161	None

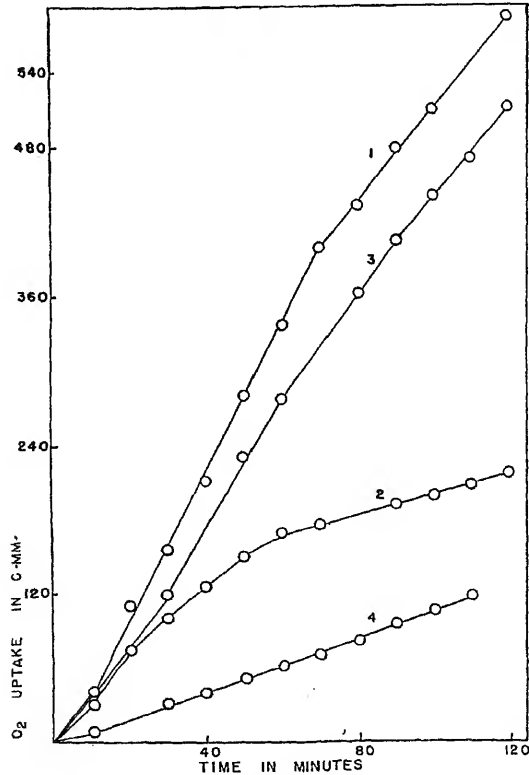


FIG. 2. Effect of FA (0.001 M) on the oxidation of pyruvate and acetate by rabbit kidney homogenates. 1. Pyruvate; 2. FA + pyruvate; 3. Acetate; 4. FA + acetate. (Blank values have been subtracted.)

observed inhibition of O_2 uptake in ground kidney with lactate as substrate is due to inhibition of an oxidation process further from pyruvate, probably acetate oxidation.

The effect of FA (0.01 *M*) on the metabolism of oxaloacetate (OA) was studied in tissue suspensions saturated with O_2 and with $N_2:CO_2$,

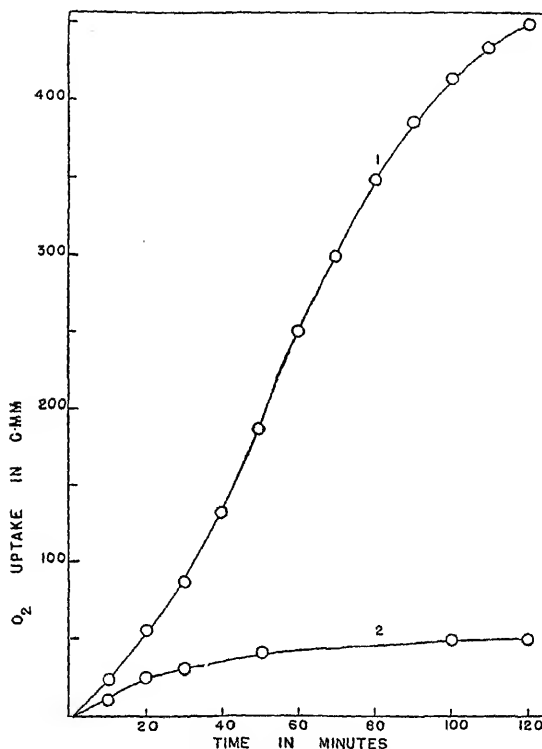


FIG. 3. The effect of FA on the oxidation of glucose by kidney homogenates. Phosphate buffer, pH 7.4; Glucose, 0.01 *M*; FA, 0.001 *M*. 150 mg. of tissue per vessel. Vol. of fluid, 3.0 cc. Temp. 38°C.

the O_2 uptake being measured in the first case and the CO_2 production in the second. FA had no effect at all (Table VIII). No difference was found in the utilization of OA on addition of FA. The oxidation of pyruvate in kidney homogenates was, as in yeast, inhibited by FA. However, measurement of the O_2 uptake at close intervals showed that

inhibition did not appear immediately, but after 20% of the pyruvate added was oxidized. This is in marked contrast with the inhibition of acetate oxidation, which takes place immediately (Fig. 2), and with the inhibition of glucose, which also occurs soon after addition of glucose (Fig. 3).

TABLE IX

Formation of Citric Acid by Rabbit Kidney Cortex Homogenates

Kidney cortex, 2 cc. containing 350 mg. tissue; BaCl₂, MgCl₂, Ba, or Mg acetate, OA, 0.12 mM. Total volume, 7 cc. Time, 45 mins. Gas phase, O₂. Temp. 38°C.

Substrates	Citric acid
	μM
None	0.94
MgCl ₂	1.1
BaCl ₂	0.86
Mg acetate + BaCl ₂	0.43
Ba acetate + BaCl ₂	0.18
OA	1.72
OA + BaCl ₂	3.48
OA + MgCl ₂	10.5
OA + Ba acetate	2.92
OA + Ba acetate + BaCl ₂	4.58
OA + Ba acetate + MgCl ₂	4.27
OA + Mg acetate + BaCl ₂	5.68
OA + Mg acetate + MgCl ₂	10.6

Effect of FA on the Oxidation of Citric Acid and on Its Synthesis

The synthesis of citric acid by yeast, which seems to occur by the condensation of acetic acid with OA, is inhibited by FA (2). The synthesis of citric acid by ground rabbit kidney cortex was studied by Wieland and Rosenthal (10). No appreciable amounts of citric acid

TABLE X

Effect of FA on the Synthesis of Citric Acid by Rabbit Kidney Homogenates

Additions	Citrate synthesis	
	Control	FA
	μM	μM
OA + Mg acetate + BaCl ₂	4.92	8.26
OA + Ba acetate + BaCl ₂	2.90	7.2
OA + BaCl ₂	3.1	4.92

were formed in the presence of acetate (Na or Ba). On the other hand, they found citric acid synthesis in the presence of acetoacetate, OA, and BaCl_2 . With the kidney homogenates, the largest amount of citric acid formed was in the presence of OA and Mg^{++} (Table IX). In fact,

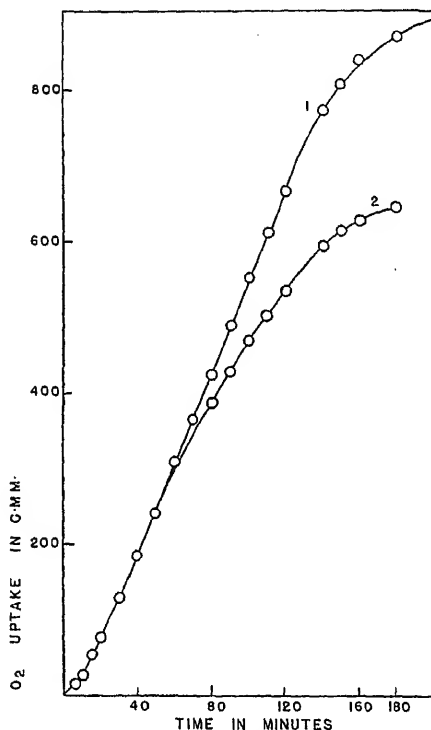


FIG. 4. Effect of FA (0.001 *M*) on the oxidation of citrate by kidney homogenates. 1. Citrate; 2. FA + citrate. (Blank values have been subtracted.)

the synthesis of citric acid by OA was 3 times as high in the presence of MgCl_2 as in the presence of BaCl_2 . Addition of Mg acetate did not increase citric acid formation.

Addition of FA, instead of inhibiting citric acid synthesis as in yeast, produced an increase (Table X). No explanation has been found yet for this increase. FA (0.001 *M*) inhibited the oxidation of citric acid. However, the inhibition started only after 10% of the citric acid had been oxidized (Fig. 4).

DISCUSSION

The experiments presented in this paper show that FA inhibition in animal tissues is less specific than the inhibition in yeast metabolism. In fact, while the oxidation of glucose by bakers' yeast was not affected by FA but after a large portion of the molecule was oxidized, it was inhibited in kidney homogenates almost completely soon after addition of glucose. In bakers' yeast FA was quite specific as an inhibitor of acetate oxidation and of citric acid synthesis; FB had no effect at all. In kidney homogenates both fluoroacids produced a powerful inhibition; indeed, the inhibitory effect of FB on the oxidation of butyrate was much greater than that of FA. Furthermore, the inhibition was not limited to acetate and butyrate oxidation; both fluoroacids inhibited strongly the oxidation of α -ketovalerate, caproate, and α -ketocaproate. This difference of action of FA was most strikingly shown on measuring the synthesis of citric acid. Citric acid formation from OA in kidney homogenates was increased in the presence of FA, although there was complete suppression of acetate utilization. Since the oxidation of citric acid and of oxaloacetic acid was not inhibited, it is difficult to interpret these findings. Undoubtedly, under the conditions of the experiments, added acetate took no part in the synthesis of citrate. Perhaps FA inhibits the formation of active acetate from acetate, and during the metabolism of OA this active acetate is formed by the oxidation of pyruvate.

SUMMARY

Fresh rabbit kidney homogenates oxidized a number of fatty acids. Acetic, butyric, β -hydroxybutyric, crotonic, vinylacetic, α -ketovaleric, methylethylacetic, caproic, and α -ketocaproic acids were oxidized vigorously, while formic, propionic, α -hydroxybutyric, α -ketobutyric were oxidized slowly. Five-hr.-old preparations largely lost this oxidizing power except for caproic and α -ketocaproic acids. FA and FB inhibited the oxidation of acetate, butyrate, α -ketovalerate, caproate, and α -ketocaproate. The oxidation of glucose was also inhibited, while the initial oxidation of citric, malic, α -ketoglutaric, and oxaloacetic acids was not affected. Pyruvate oxidation was inhibited. FA inhibition of acetate oxidation was not released by ethyl alcohol.

The synthesis of citric acid in the presence of OA was greatly increased on addition of Mg^{++} . This synthesis was increased by FA, although acetate utilization was completely suppressed.

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On the Activation of Lipoxidase

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INTRODUCTION

In 1943 Balls, Axelrod and Kies (1) reported that soy beans, barley malt, rice polishings, various legumes, yeast, rabbit heart muscle, beef muscle, and milk contained a substance which activated lipoxidase. They were able to concentrate the material from soy beans to a considerable extent and considered it to consist largely of peptides. Theorell, Bergström and Åkeson (8), in 1944, also claimed to have obtained a substance of protein nature from soy bean meal which would activate lipoxidase. They called this substance "the activating enzyme." However, Cosby and Sumner (4, 5) were not able to demonstrate the presence of an activator in soy beans, or in any of the other materials mentioned by Balls *et al.*

In 1946, Balls *et al.* (2) repeated their claim for the existence of an activator and claimed that failure of Cosby and Sumner to detect this substance was due to the use of gum arabic in their test solution. They stated that their activator functioned as a stabilizer and not as a coenzyme. Recently Kies (7) has reiterated the claims made by Balls *et al.* and has isolated a new activator from soy bean meal which is not identical with the old activator.

In a recent paper by Theorell *et al.* (10) on the preparation of crystalline lipoxidase, they state, "this homogeneous enzyme preparation does not need any activator under our assay conditions." They, however, have given no data to substantiate their claims. This statement has also been reiterated by Holman (6), who has continued the studies on the properties of the enzyme prepared in Theorell's laboratory.

It is hard to correlate these statements with those of other workers, since no data have been presented to substantiate their claims and since the conditions under which the enzyme activity was determined produce marked changes in the apparent activity of the preparation which obscures the true kinetics of the reaction.

It has been repeatedly shown that the optimum conditions for lipoxidase activity are pH 6.5 and 25°C. (11, 12, 13). If either the pH value or the temperature of the test solution is increased, there will be a marked decrease in enzymatic activity. This effect, however, will

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only be noticeable if the substrate used exists in a chemical form, such as methyl linoleate, which does not undergo changes in its physical state with changes in pH (11). If, on the other hand, a substrate such as sodium linoleate is used, which changes from a colloidal phase to a soluble phase with increase in pH value (11), there will be an increase in the apparent activity of the enzyme preparation, due to an increase in the availability of the substrate.

Since both Theorell *et al.* (9), and Holman (6), have used sodium linoleate and high pH values (pH 9), it is hard to evaluate their statements in view of the fact that 3 separate factors, namely, the effect of pH on decreasing the true activity of the enzyme, the effect of pH on increasing the availability of the substrate, which results in an apparent increase in enzyme activity, and finally, the effect of the activator, are influencing simultaneously the observed activity of the enzyme preparation.

EXPERIMENTAL

We have prepared the activator of Balls *et al.* (1) and the activating enzyme of Theorell *et al.* (8) according to the methods described by the respective authors. Both activators have been tested with biuret, Millon's, Hopkins-Cole, and ninhydrin rea-

TABLE I

The Effect of Magma of Bentonite-N.F. on the Rate of Decolorization of a Fatty Acid-Bixin-Lipoxidase System

Sample	Magma of bentonite added ml.	Time for decolorization secs.
I	0.00	60
II	0.10	40
III	0.13	27
IV	0.15	26
V	0.18	38

TABLE II

The Effect of Sodium Dodecyl Sulfate^a on the Rate of Decolorization of a Fatty Acid-Bixin-Lipoxidase System

Sample	Sodium dodecyl sulfate added ml.	Time for decolorization secs.
I	0.00	14
II	0.05	8
III	0.10	6
IV	0.15	8
V	0.20	10
VI	0.30	13

^a Approximately 3 mg./ml.

gents and their ability to absorb light in the ultraviolet region from 230 $m\mu$ to 300 $m\mu$ have been determined. The results of these tests agree with those given by Balls *et al.* for their activator and they indicate that the "activating enzyme" of Theorell *et al.* is probably of similar nature.

The bixin decolorization method (13) has been employed to determine the effect of both activators on a highly purified lipoxidase preparation. Since Balls has objected to the use of gum arabic in this type of test on the basis that the activator, and possibly lipoxidase itself, were present in gum arabic, various emulsifying agents were tested to find a stabilizing substance which would maintain the fatty acids and bixin in a finely dispersed state. If the bixin, fatty acids, and phosphate buffer are mixed in the absence of a stabilizing or emulsifying agent, the fatty acids separate from the water phase. This in turn removes the major portion of the bixin, since it is more soluble in the fatty acid phase than in the water phase. This causes an apparent decrease in the activity of the lipoxidase preparation by removing the substrate from contact with the enzyme.

TABLE III

The Effect of Soy Bean Activator on an Emulsified System of Fatty Acid-Bixin-Sodium Dodecyl Sulfate-Lipoxidase

Sample	Sodium dodecyl sulfate added	Soy bean activator added	Time for decolorization
	<i>ml.</i>	<i>ml.</i>	<i>secs.</i>
I	0.0	0.0	14
II	0.1	0.0	7
III	0.1	0.05	10
IV	0.1	0.10	11
V	0.1	0.20	11

TABLE IV

Digest Set-Up for Studying the Oxygen Uptake with Activator-Lipoxidase Systems in the Warburg Apparatus

Reagent	Flask no.				
	1	2	3	4	5
			<i>ml.</i>		
Water	2.3	—	—	0.1	0.1
Lipoxidase (side bulb No. 1)	—	0.1	0.1	0.1	0.1
Fatty acids substrate	—	2.0 ^a	2.0 ^a	2.0	2.0 ^a
20% KOH + inset	—	0.1	0.1	0.1	0.1
Activator (side bulb No. 2)	—	0.1	0.1	—	—

^a Fatty acids substrate containing 0.1 ml. of activator/2 ml. of substrate. Substrate prepared according to the method of Cosby and Sumner (5).

We have found that both bentonite and sodium dodecyl sulfate possess a stabilizing action, thereby increasing the rate of decolorization of bixin by the lipoxidase system (Tables I and II). Here, neutralized soy bean fatty acids, bixin, phosphate buffer of pH 6.5, and highly purified soy bean lipoxidase were employed. Neither bentonite nor sodium dodecyl sulfate could possibly show lipoxidase activity or contain the activators described by Balls *et al.* or Theorell *et al.* If these activators have an activating or stabilizing effect on the lipoxidase, it should be possible to demonstrate the action, using a system

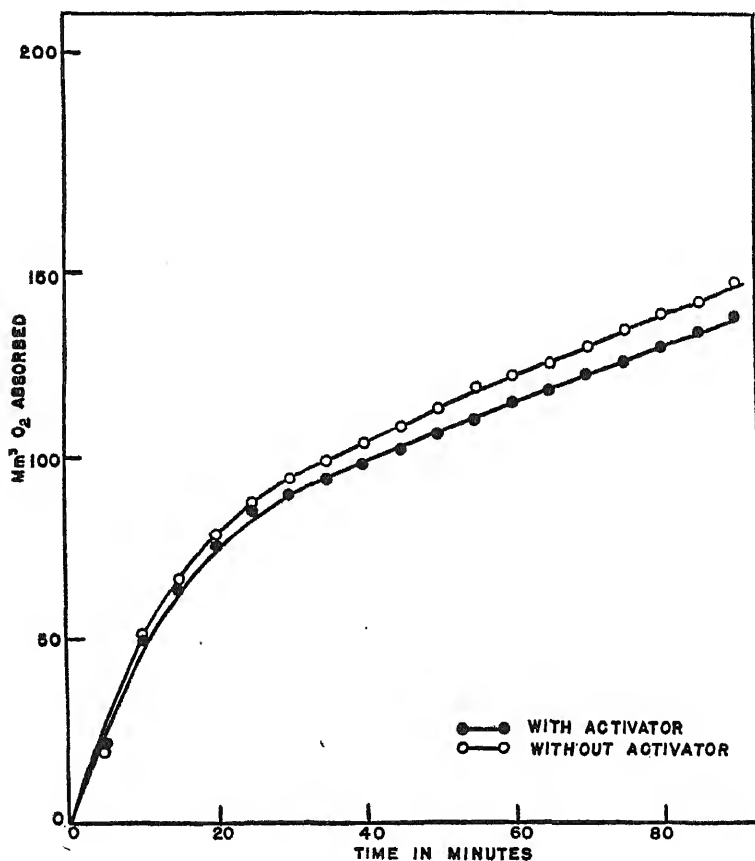


FIG. 1. Oxygen absorption of lipoxidase-fatty acid systems with and without Balls *et al.* activator.

previously emulsified by sodium dodecyl sulfate or bentonite. As is shown in Table III, the addition of the Balls *et al.* soy bean activator, is here entirely without effect. Furthermore, a slight decrease in activity was noticed, which parallels the results obtained by Balls *et al.* when an excess of their activator was added to their test solution.

Recently Dr. T. C. Chou (3) has tested the new activator of Kies (7), using neutralized soy bean fatty acids, bixin, phosphate buffer of pH 6.5, and purified lipoxidase. He reports that the Kies activator, as prepared by him, was partly crystalline and possessed slight lipoxidase activity. Upon heating to 95°C. for 10 mins. all lipoxidase activity was lost. The activator then failed to accelerate the bleaching of bixin.

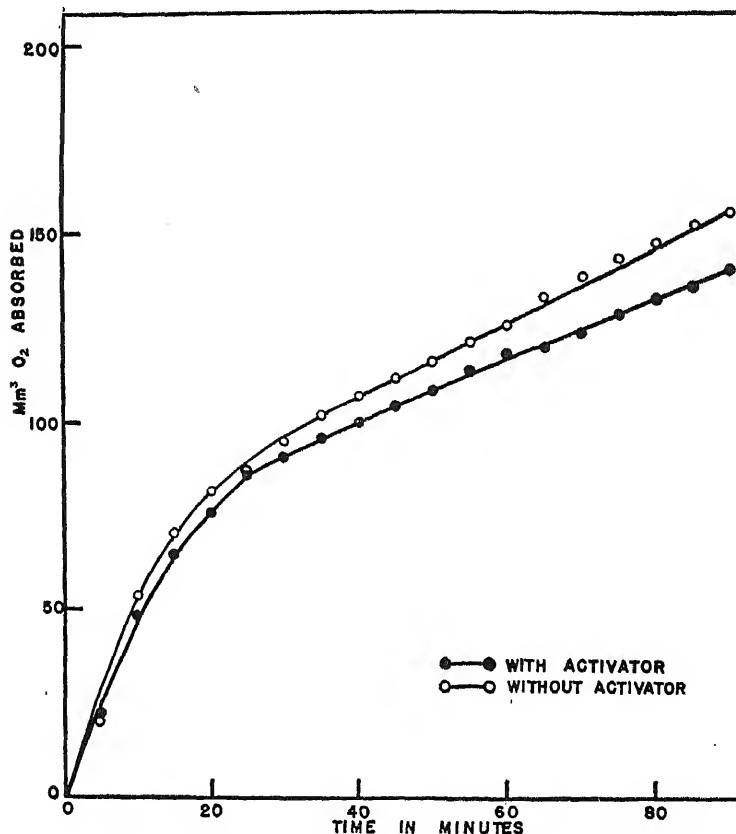


Fig. 2. Oxygen absorption of lipoxidase-fatty acid systems with and without Theorell *et al.* activating enzyme.

We have tested the activators of both Balls *et al.* and of Theorell *et al.* by measuring the uptake of oxygen, using the Warburg apparatus, during the oxidation of a fatty acid substrate by lipoxidase. Here the digests contained the materials indicated in Table IV. It will be noted that flasks No. 2, 3, and 5 contained the Balls *et al.* or Theorell *et al.* activator at the start, while flask 4 contained none. The oxygen absorption was determined at 5 min. intervals for 60 mins. after the addition of the lipoxidase. Second portions of the activator, present in side bulbs No. 2 were then added to the main portions of flasks Nos. 2 and 3 and the readings continued for another 30 mins. Data from these determinations are plotted in Figs. 1 and 2. There were no increases in oxygen absorption when either activator was present at the start, or added later. Similar results have been obtained using bentonite as the emulsifying agent.

SUMMARY

The activator of Balls *et al.* (1, 2), the activating enzyme of Theorell *et al.* (8), and the new activator of Kies (7), have been tested in the lipoxidase system, using the bixin decolorization and the oxygen absorption methods. No activation could be demonstrated. Both bentonite and sodium dodecyl sulfate have been found to exert a stabilizing action upon the lipoxidase system.

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Identification and Assay of Monamine Oxidase in the Human Placenta

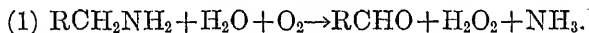
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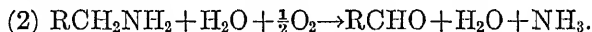
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INTRODUCTION

In 1910 Ewins and Laidlaw (1) isolated *p*-hydroxyphenylacetic acid from a solution of tyramine perfused through the liver, thus demonstrating the ability of an organ of the body to deaminate and oxidize an amine. Hare (2) in 1928, described the enzyme, tyramine oxidase, responsible for this reaction. Blaschko *et al.* (3) found that tyramine oxidase, adrenaline oxidase, and the aliphatic amine oxidase of Pugh and Quastel (4), are the same enzyme. This enzyme has been called monamine oxidase to distinguish it from the diamine oxidase of Zeller (5). Kohn (6) showed that monamine oxidase catalyzes the following reaction:



Since the peroxide formed is decomposed by catalase the net reaction is:



The aldehyde can be oxidized further to an acid by Schardinger's enzyme (7) with the uptake of one additional atom of oxygen. There is also the possibility that the two moles of aldehyde may undergo a Cannizzaro reaction, catalyzed by aldehyde mutase, to form the corresponding acid and alcohol. This type of reaction occurs with butylamine and isoamylamine (8).

Monamine oxidase has been found to occur in the liver, intestine, uterus, kidney (9), brain (4), prostate, and sperm (10). Page (11) observed oxygen uptake with placental extract in the presence of tyramine. The present paper is concerned with the demonstration of the presence of monamine oxidase in the placenta and with the development of methods for its quantitative assay in that tissue. Some substrates of monamine oxidase are vasopressor substances with a sympathomimetic effect (12) and their

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deamination, for example that of oxytyramine, is accompanied by a loss of pharmacological activity (13). Therefore, it has been suggested (14) that the enzyme is the physiological inactivator of the sympathomimetic transmitter substance. Raska (15) found that monamine oxidase activity of the kidney is reduced in dogs with experimental hypertension produced by partial obliteration of the renal artery.

Therefore, it seemed possible that changes in the activity of monamine oxidase in the placenta could be concerned with the etiology of toxemia in late pregnancy, which has been ascribed to placental ischemia.

METHODS

The enzymatic activity was measured by following the oxygen consumption in air in the Warburg apparatus at 38°C. Immediately after delivery the placentae were placed in the refrigerator and used within 2 hrs. Before use, the maternal surface was washed free of blood with cold running tap water and wiped with filter paper. The tissue was cut parallel to the maternal surface and about 0.5 cm. away from it. The desired amount was weighed and homogenized at a temperature of 10–18°C. with 4 volumes of *M*/20 phosphate buffer, pH 7.4, unless otherwise indicated (3, 13). The reaction volume was kept under 2.5 cc. of which 2 cc. were homogenate. The representative substrates employed were tyramine-HCl, tryptamine-HCl, isoamylamine and butylamine, the latter two adjusted to pH 7.4. The center cup contained 0.2 cc. of 2 *N* KOH and filter paper. The experiments were run for one or more hours, as noted.

The determinations of ammonia were carried out by a modified Conway procedure (16). At the end of the experiment, the reaction was stopped by the addition of 0.2 cc. of 25% trichloroacetic acid. The contents of the vessels were removed and centrifuged. A 1 cc. aliquot of the supernatant was introduced into the outer Conway chamber. The inner chamber contained 1 cc. of a 1% boric acid solution and 0.01 cc. of Ma's indicator (17). One cc. of 50% KOH was then added to the outer chamber and the cover closed. After 2 hrs., the ammonia contained in the boric acid of the inner chamber was titrated with *N*/100 potassium biniodate solution.

RESULTS

Properties of Enzyme

The use of an homogenate in preference to an extract seemed to be indicated, in order to obtain a quantitative estimation of monamine oxidase activity. A crude extract was prepared by centrifugation of the homogenate. It was found that with tyramine-HCl, in *M*/72 final concentration, the homogenate caused an oxygen uptake of 117 and the extract 93 mm.³/hr. The substrate blanks were 2 and 0 mm.³, respectively. Other experiments, in which the oxygen uptake of the residue

was measured, demonstrated that some activity remained unextracted. It therefore appeared that the most reliable quantitative data could be obtained by the use of tissue homogenates.

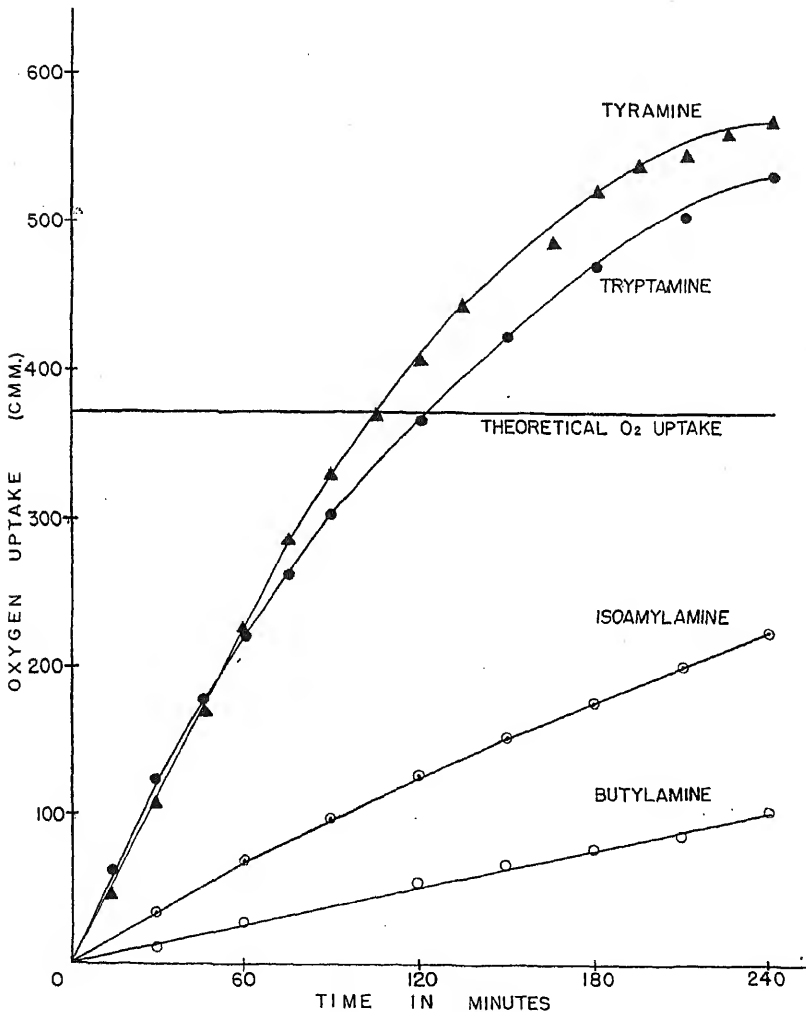


FIG. 1. Oxidation of various amines by placental homogenate. Each vessel contained 2.0 cc. of placental homogenate (0.25 g./cc.), 0.2 cc. of *M*/6 amine and water to a final volume of 2.3 cc., alkali in the center-cup. The substrate blanks were negligible and therefore not drawn. The horizontal line represents the theoretical oxygen uptake for Eq. 2.

Monamine oxidase attacks a large number of aliphatic and aromatic amines provided they have a terminal NH_2 group (3). Placental homogenate showed oxygen uptake in the presence of tyramine, tryptamine, butylamine, and isoamylamine, but the reaction velocities differed widely (Fig. 1). Butylamine showed the slowest, but most constant, rate over a period of 4 hrs. The oxygen uptake with tyramine and tryptamine exceeded the theoretical value of 374 mm.³, which is equivalent to the complete deamination of 0.2 cc. of $M/6$ amine, if only 1 g.-at. of oxygen reacts (Eq. 2). The explanation for this excess is discussed below.

If the relative deamination velocities found with placental homogenate are compared with those obtained by Blaschko *et al.* (3) with guinea pig liver extract, similar velocities are only found for the aromatic amines (Table I). The aliphatic amines, however, were found to

TABLE I

Comparison of Monamine Oxidase Reaction Rates of Placenta and Guinea Pig Liver
Duration of exp., 1 hr.; oxygen uptake of tyramine equal to 100

	Placenta	Guinea pig liver Blaschko <i>et al.</i> (3)
Tyramine	100	100
Tryptamine	97	87
Butylamine	12	54
Isoamylamine	30	105

be oxidized more slowly by placenta than by liver. Since crude enzyme preparations were employed in both cases, it is extremely unlikely that the differences in rates indicate a qualitative difference in monamine oxidase activity. They may more justifiably be ascribed to the presence in liver extracts of enzymes not present in placenta, which catalyze the further oxidation of the aliphatic aldehydes initially formed.

The effect of enzyme concentration upon reaction velocity was investigated to determine the range in which the rate of oxygen uptake is directly proportional to enzyme concentration. For the substrates tested, this range extends from 0.075 g. of placental tissue/cc. homogenate to 0.25 g./cc. (Fig. 2). The reaction was of zero order for 1 hr. within these limits. The absence of direct proportionality for homogenate concentrations higher than 0.25 g./cc. was not due to lack of substrate. Investigation of substrate dependence showed enzyme saturation at a final concentration of substrate as low as $M/90$. Conse-

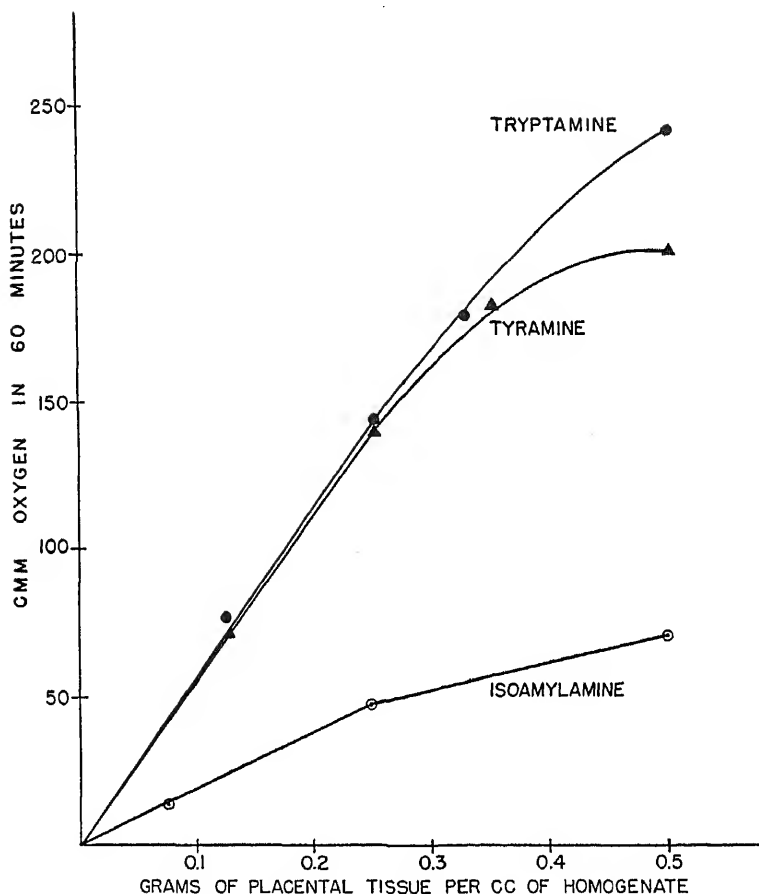


FIG. 2. Rate of oxygen uptake as a function of the concentration of the enzyme. Each vessel contained 2.0 cc. of placental homogenate, 0.2 cc. of *M/6* amine and water to a final volume of 2.3 cc.; alkali in the centerwell. The curve for tyramine is from a different placenta. Reaction time 1 hr. The substrate blanks were negligible (3 and 5 mm.³/hr).

quently, in a quantitative assay of the activity of monamine oxidase in the placenta, a maximum of 2.0 cc. of homogenate containing 0.25 g. of tissue/cc. may be used with 0.15 cc. of *M/6* amine for a 1 hr. period.

Other factors were studied which might influence the enzymatic activity. The enzyme is fairly stable. A decrease of about 10% in

oxygen uptake was observed in homogenates kept at 5°C. for 16 hrs. A similar loss of activity was encountered if the homogenate was kept at 38°C. for 2 hrs. Bacterial contamination can not be excluded when the placenta is delivered normally. Placentae obtained by Caesarean section, however, gave essentially the same values. The type of anesthesia used during the delivery did not influence the enzymatic activity.

Reaction Products

The demonstration of the reaction products, aldehyde, peroxide, and ammonia, provides additional proof for the presence of monamine oxidase in the placenta. The formation of aldehyde was demonstrated following the technique of Kohn (6) and Raska (15). Twenty cc. of placental homogenate (0.25 g./cc.) were incubated for 90 mins. at 38°C. in air with 2.0 cc. of *M*/6 tyramine-HCl under vigorous stirring. The reaction was stopped by the addition of 2.0 cc. of 25% trichloroacetic acid and the proteins removed by centrifugation. Ten cc. of the supernatant were added to an equal volume of a saturated solution of 2, 4-dinitrophenylhydrazine in *N* HCl. There was an immediate formation of a fine yellow precipitate which increased on standing in ice overnight. The formation of this precipitate indicates the presence of an aldehyde in the mixture (6).

The formation of peroxide during deamination by monamine-oxidase was proved by Kohn (6). Keilin and Hartree (18) showed that, in the presence of catalase, ethyl alcohol is oxidized to an aldehyde by the peroxide formed during the oxidation of hypoxanthine by xanthine oxidase. Kohn (6), using this observation, found that the addition of ethyl alcohol to the monamine oxidase-catalase system almost doubled the oxygen consumption.

The oxygen uptake at 30°C. with placental homogenate was increased 59% in the presence of alcohol, whereas at 38°C. the increase was only 9% (Table II). Therefore, we can conclude that peroxide is formed during deamination by the placenta. The smaller increase of the

TABLE II
*Effect of Ethyl Alcohol on the Oxygen Uptake by Placental
Homogenate in the Presence of Tyramine*

	Oxygen uptake in mm. ³	
	at 38°C.	at 30°C.
Without alcohol	270	92
With alcohol	294	146
Increase per cent	9	59

The following additions were made: 2.0 cc. of homogenate (0.25 g./cc.), 0.2 cc. of *M*/6 tyramine-HCl, 0.1 cc. of 10% ethyl alcohol or water to a final volume of 2.3 cc. Alkali in centerwell; reaction time 1 hr.

oxygen uptake with alcohol at 38°C. might be explained by the more rapid inactivation of catalase at the higher temperature.

Further evidence of the formation of peroxide can be deduced from the fact that, in all experiments, the color of the homogenate changed from red to brown a few minutes after addition of the substrate. According to Kohn (6), this brown color is due to methemoglobin formed in the presence of peroxide. Holtz (13) assumes, however, that this pigment is due to quinone formation from tyramine. In the experiments reported here, the brown color occurs with the aliphatic amines as well as with tyramine. Furthermore, typical absorption bands for alkaline methemoglobin were seen. These observations suggest that a part of the peroxide formed during deamination escapes decomposition by catalase and is used for the oxidation of hemoglobin. It would appear that this formation of methemoglobin will cause the oxygen uptake to exceed the theoretical amount required by Eq. 2. Moreover, the further oxidation of aldehyde is another factor which may contribute to oxygen utilization beyond the theoretical requirements. Indeed, excess of oxy-

TABLE III

Effect of Semicarbazide on Oxygen Uptake and Ammonia Production by Placental Homogenate in the Presence of Tyramine

Placenta sample no.	NH ₃ production	O ₂ consumption		Ratio O:NH ₃	
		Without	With	Without	With
		Semicarbazide		Semicarbazide	
	μM	atoms.			
829	10.4	15.4	11.4	1.48	1.10
94	10.0	14.0	10.5	1.40	1.05
118	13.0	17.1	13.5	1.32	1.04
1121	12.6	17.1	13.4	1.36	1.06
1218	12.1	17.2	13.5	1.42	1.11
1222	12.1	16.2	13.8	1.34	1.14
122	12.4	17.7	13.8	1.43	1.11
Av:				1.39	1.09

The following additions were made: 2.0 cc. of homogenate (0.25 g./cc.), 0.15 cc. of *M*/6 tyramine-HCl, 0.15 cc. of *M*/3 semicarbazide adjusted to pH 7.4 or water to a final volume of 2.3 cc. Alkali in centerwell; reaction time 1 hr. The values are corrected for blank respiration and preformed tissue—NH₃.

gen uptake has been observed when calculated on the basis of complete oxidative deamination (Fig. 1). It is also illustrated by a comparison of the oxygen uptake and the ammonia production. Table III shows that the oxygen uptake exceeds the theoretical ratio of 1 atom of oxygen per mole of ammonia. The observed ratio in the absence of semicarbazide is 1.39. This table shows that fairly reproducible ratios can be obtained.

Inhibitors

The study of inhibitors was undertaken for a twofold purpose: 1, the exclusion of other enzymes as the cause of oxygen consumption in the presence of the substrate used; and 2, the prevention of further oxidation of reaction products. Tyramine can be oxidized on the ring by polyphenoloxidase (19) and the cytochrome system (20). These enzymes are sensitive to cyanide. According to Hare (2,7) monamine oxidase in an alkaline medium is not inhibited by 0.005 *M* cyanide. Hare's observations were made without providing an equilibrium cyanide concentration in the center well. Indeed, if the centercup is left empty or contains only alkali, 0.005 *M* KCN does not cause any inhibition of oxygen uptake or decrease in ammonia formation with placental homogenate. It is to be expected that under these conditions cyanide will be liberated to the gas phase of the vessel and also be bound in increasing amounts during the reaction by various substances (*e.g.*, the aldehyde formed, hemoglobin, *etc.*) and, therefore, the original cyanide concentration will fall off progressively. However, inhibition was observed in this study when precautions are taken to maintain the KCN concentration by adding cyanide to the alkali in the centerwell following the technique of Umbreit (21). Under such conditions, the inhibition started after 20 mins. and increased progressively thereafter. These observations are given to show that under physical conditions, similar to those of Hare, the same effect is obtained with placenta, as by Hare with liver. However, the facts presented suggest that the cyanide effect upon impure monamine oxidase systems is more complicated and further investigations in respect to its mechanism are at present in progress.

Monamine oxidase is destroyed by octyl alcohol. Holtz and Heise (13) found 84% inhibition of monamine oxidase in kidney extracts with octyl alcohol. Sixty per cent inhibition was found with placental homogenate under similar conditions. The inhibition by octyl alcohol is

additional evidence in favor of the identification of the enzyme as being monamine oxidase.

Further proof for the presence of monamine oxidase in the placenta was established by the study of the effect of semicarbazide. If placental homogenate reacts with tyramine, the ratio $O:NH_3$ was 1.39 after 1 hr. period (Table III). The formation of methemoglobin, the oxidation of the aldehyde formed during the deamination and the oxidation on the ring of tyramine by other enzymes were mentioned as the most likely causes of this ratio. The oxidation of *p*-hydroxyphenylacetaldehyde to the corresponding acid can be prevented by the addition of semicarbazide (22). This was confirmed for placental homogenate. The addition of semicarbazide adjusted to pH 7.4 in a concentration twice that of amine decreases the ratio $O:NH_3$ to 1.09 (Table III). This change in ratio with a constant ammonia production is indicative of the fixation of the aldehyde formed and characterizes the observed process as one of oxidative deamination with formation of a carbonyl-containing compound. The mechanism of action of semicarbazide was further demonstrated by following the oxidation of 0.05 cc. of *M*/6 tyramine to completion (Fig. 3). Without semicarbazide the oxygen consumption was 140 mm.³ (Curve 3), with semicarbazide in a concentration 2–8 times that of amine it was 111 mm.³ (Curves 4 and 5), *i.e.*, 17 mm.³ in excess of the 94 mm.³ required by Eq. 2 for this amount of substrate. This difference of 17 mm.³ may be due to the formation of methemoglobin by the peroxide formed during the deamination. The controls with larger amounts of substrate prove that there was no deterioration of enzymatic activity during the experimental period (Curve 1) and that semicarbazide does not act by inhibiting the enzyme (Curve 2); were such the case there should be no higher uptake of oxygen than in the experiment of Curve 5.

Bernheim *et al.* (23) estimated that, when catalase and hemoglobin compete for peroxide, the methemoglobin formed represents about 10% of the peroxide formed by the tissues. The average ratio of 1.09 found in the presence of semicarbazide with placental homogenates after a 1 hr. period (Table III), and the ratio of 1.18 (111/94) observed by following the oxidation to completion (Fig. 3), are in agreement with Bernheim's estimation and with the theory for Eq. 2. The oxidation of aldehyde and hemoglobin combined account completely for the oxygen excess of 0.39 and therefore exclude any ring oxidation of tyramine by other enzymes as a possible cause of this excess. The ratio of 1.09 can be

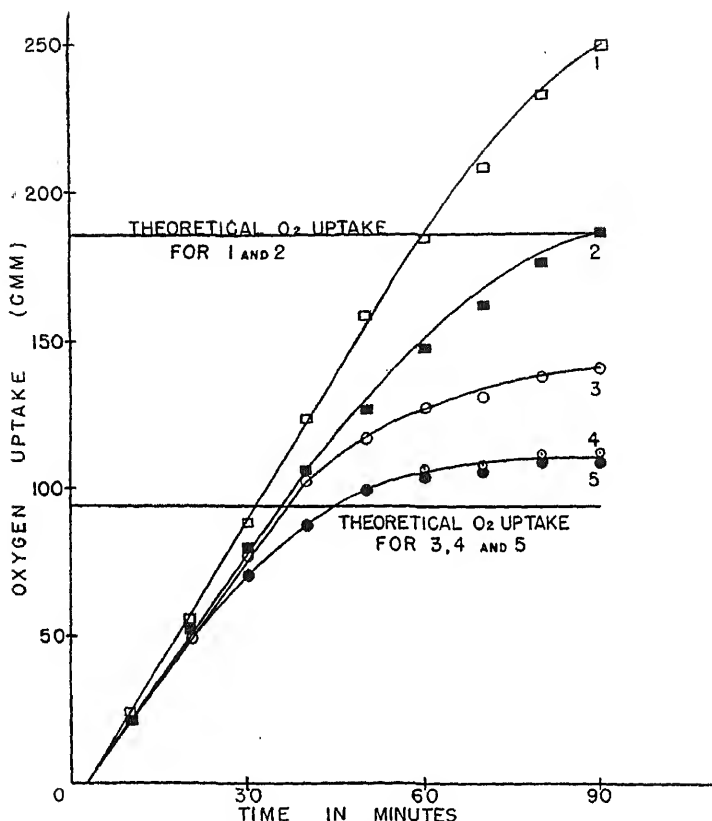


FIG. 3. Effect of semicarbazide on the oxygen uptake of placental homogenate in the presence of tyramine. Each vessel contained 2.0 cc. of homogenate (0.25 g./cc.); in addition, vessels 1 and 2 contained 0.1 cc., and vessels 3, 4, and 5 0.05 cc., of $M/6$ tyramine-HCl; vessels 2, 4, and 5 contained, respectively, 0.2, 0.05, 0.2 cc. of $M/3$ semicarbazide adjusted to pH 7.4; water was added where necessary to a final volume of 2.3 cc.; alkali in the centercup. The horizontal lines represent the theoretical oxygen uptake for Eq. 2. The substrate blanks were negligible.

obtained only if deterioration of catalase is reduced to a minimum by using the placenta within 2 hrs. after its delivery. Under these conditions, the addition of semicarbazide allows reproducible correlation of oxygen consumption with ammonia production. The NH_3 blanks for 2 cc. of homogenate (0.25 g./cc.) were relatively constant, amounting to 15–20 γ of $\text{NH}_3\text{-N/cc.}$ of homogenate. Therefore, the experimental con-

ditions for the quantitative assay of monamine oxidase activity in placental homogenate may be defined as those contained in the legend of Table III with the addition of another submaximal enzyme level.

The identity of the enzyme can be further demonstrated by competitive inhibition. Phenylisopropylamine and its derivate, ephedrine, are not oxidized by monamine oxidase, but ephedrine shows competitive inhibition with *l-p*-sympathol (24) and isoamylamine (3) as substrates. This was confirmed for placental homogenates. *dl*-Ephedrine-HCl did not show any oxygen uptake with placental homogenate. However, in a concentration 5 times that of tyramine, it inhibited 33%, and in a concentration 8 times that of isoamylamine the inhibition amounted to 66% (Table IV).

TABLE IV

Effect of Addition of Ephedrine on the Oxygen Uptake of Placental Homogenate in the Presence of Various Substrates

Substrate (molar concentration)		Oxygen Consumption				Inhibition
		Ephedrine (molar concentration)				
		Zero	M/55	M/138	M/69	
Tyramine	M/138	mm. ³ 156	mm. ³	mm. ³ 140	mm. ³	percent 10
	M/276*	152*	100*			33
Isoamylamine	M/138	34		17		50
	M/552	24			8	66

The following additions were made: 2.0 cc. of homogenate (0.25 g./cc.), amines and ephedrine to above final concentrations, water to a final volume of 2.3 cc.; alkali in the center cup. Reaction time 1 hr. Tissue blanks were subtracted. In the experiment marked by * the hourly uptake was calculated from the 40 min. uptake.

When a mixture of 0.1 cc. of tyramine and 0.1 cc. of tryptamine was used to study substrate competition, no additional oxygen uptake occurred. During a 30 min. period 2.0 cc. of homogenate (0.25 g. of tissue/cc.) caused an uptake of 78 mm.³ with 0.1 cc. of tyramine-HCl as substrate, 88 mm.³ with 0.1 cc. of tryptamine-HCl and 80 mm.³ with the mixture. Therefore, the same enzyme is concerned with the deamination of both.

DISCUSSION

The role of monamine oxidase in the placenta is subject to speculation. If about 672 γ of tyramine are deaminated by 0.5 g. of placental

tissue/hr., then, theoretically, about 0.7 g. of tyramine can be deaminated by the average placenta/hr. It is probable that in the placenta the monamine oxidase serves in the detoxification of amines. Inasmuch as some amines are sympathomimetic in their vasomotor action, monamine oxidase in the placenta may separate the fetus from maternal vasoactive substances and, conversely, the mother from those in the fetus. The activity of monamine oxidase depends upon partial oxygen tension (6). Therefore, it is permissible to assume that, under conditions of placental ischemia (25), the enzyme may not fulfil its physiological role: destruction of vasopressor amines. This question is at present being investigated in our laboratory.

SUMMARY

Monamine oxidase is contained in the human placenta at term. The presence of this enzyme was proved by the study of substrate specificity, the demonstration of the reaction products, and the action of inhibitors.

Methods for the quantitative estimation of the activity of monamine oxidase were tested for placenta with the following results: 1, homogenate is preferable to extract as source of enzyme; 2, the quantitative determination of ammonia yields a more accurate estimate of oxidative deamination than the measurement of oxygen consumption alone; 3, the partial exclusion of secondary oxidations by semicarbazide results in a reproducible $O:NH_3$ ratio close to the theoretical value of 1.00.

A discussion is presented of the possible role of this enzyme in pregnancy.

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Niacin Deficiency Anemia in Swine ¹

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INTRODUCTION

The role of niacin in erythropoiesis has been obscure. The association of anemia with pellagra has long been recognized, but proof that the anemia is due to niacin deficiency is lacking (1). Earlier investigators described anemia and leukopenia in various animals receiving black tongue-producing diets or diets lacking in nicotinic acid (2, 3, 4), but the relation of niacin to erythropoiesis was not clearly shown until Handler and Featherston (5) reported that the parenteral administration of physiological saline solution to dogs with acute black tongue alleviated the existing hemoconcentration and was associated with the appearance of severe anemia. The anemia responded specifically and rapidly to the administration of synthetic nicotinic acid. The anemia did not respond to the administration of highly purified liver extract and was reported to be either macrocytic or normocytic. The bone marrow was hypoplastic. The authors postulated that, since immature nucleated erythrocytes respire, they probably require pyridine nucleotides. Consequently, as the supply of nicotinic acid diminished, anemia developed owing to a lack of cozymase in the earliest stages of cell development.

Recently, Elvehjem and his associates (6, 7) have reported that young dogs fed niacin-deficient rations but supplemented with adequate amounts of thiamine, riboflavin, pyridoxine, pantothenic acid, choline, pteroylglutamic acid, and biotin, developed macrocytic anemia which was not affected by niacin but did respond to purified liver extract.

Earlier investigations from this laboratory (8) demonstrated that swine maintained on a low-protein (10% casein), niacin-deficient diet

¹ Aided by a grant from the U. S. Public Health Service, and by grants from the Upjohn Co., and Parke, Davis and Co.

developed signs of nutritional deficiency and a severe anemia. Since infections occurred in these animals, no conclusions were drawn regarding the significance of the anemia.

In view of the somewhat conflicting experiments in dogs mentioned above it was decided to study the effect of niacin on erythropoiesis in swine and to determine whether repeated niacin depletion gives rise to a deficiency of a substance contained in highly purified liver extract.

EXPERIMENTAL PROCEDURE

For this study 20 weanling pigs, 21–28 days of age, were used. Eight animals received niacin in the supplement and served as controls. Twelve animals were given diets without added niacin. The animals were placed on either a low-protein, high-fat diet consisting of Sheffield's "New Process" casein, 10%; sucrose, 57.7%; lard, 27.1%; and swine salt mixture No. 3 (9), 5.2%; or a low-protein, high carbohydrate diet consisting of casein, 10%; sucrose, 73.8%; lard, 11.0%; and swine salt mixture, 5.2%. In addition, all animals received cod liver oil (Mead Johnson, 1800 units of vitamin A, 175 units of vitamin D/g.), 0.5 g./kg. of body weight daily or Natola (Parke Davis, 55,000 units of vitamin A, 11,000 units of vitamin D/g.), 0.056 g./kg. of body weight per week. Vitamins were supplied in crystalline form by placing them in capsules and administering them orally 3 times a week. The quantities of crystalline vitamins, when these were given, were as follows (mg./kg. of body weight daily):

TABLE I
Basal Diet and Vitamin Supplements

Animals	Basal diet	Vitamin supplement
9-61 to 9-63 inclusive	Low-protein-high fat	T, R, N, B ₆ , P, C, I, PABA
10-45 to 10-50 inclusive	Low-protein-high fat	T, R, N, B ₆ , P, C, ^a I, PABA, B, PGA
9-58 to 9-60 inclusive	Low-protein-high fat	T, R, B ₆ , P, C, I, PABA
10-36 to 10-38 inclusive	Low-protein-high CHO	T, R, B ₆ , P, C, B
10-39 to 10-44 inclusive	Low protein-high CHO	T, R, B ₆ , P, C, B, PGA ^b

T—thiamine

R—riboflavin

N—nicotinic acid

B₆—pyridoxine

P—pantothenic acid

C—choline

I—inositol

PABA—*p*-aminobenzoic acid

B—biotin

PGA—pteroylglutamic acid

^a Choline chloride, 0.50 mg./kg. of body weight daily.

^b Pteroylglutamic acid increased to 100 mg. daily on 168th day of experiment.

TABLE II
Hematologic Data on 12 Low-Protein—Niacin-Deficient Animals

Pig	Days on exp.	RBC mill./mm. ³	Hgb g-%	Ht ml./100 ml.	MCV μ^3	MCH $\gamma\gamma$	MCHC per cent	Reties per cent	WBC $\times 1000$ mm. ³	I.I.
9-58	115	3.77	6.7	22.4	60	18	30	5.4	14.4	2
9-59	97	4.35	6.2	23.0	53	14	27	5.0	19.7	3
9-60	73	4.22	7.5	23.0	55	18	33	7.8	20.5	2
10-36	55	5.17	9.0	29.0	56	17	31	1.4	12.2	2
10-37	58	3.68	8.1	25.8	70	22	31	2.6	16.3	2
10-38	58	3.58	6.9	22.8	64	19	30	0.8	12.1	2
10-39	55	4.79	8.3	28.2	59	17	30	0.4	14.9	2
10-40	58	5.96	9.2	28.8	48	15	32	0.2	20.7	2
10-41	58	4.79	7.5	25.0	52	16	30	1.6	10.3	2
10-42	94 ^a	3.65	6.2	21.0	58	17	30	1.2	19.6	2
10-43	94 ^a	3.44	5.9	18.8	55	17	31	1.4	14.6	5
10-44	94 ^a	4.03	6.6	23.0	57	16	29	3.6	22.1	5

Ht—volume of packed red cells.

MCV—mean corpuscular volume.

MCH—mean corpuscular hemoglobin.

MCHC—mean corpuscular hemoglobin concentration.

I.I.—icterus index.

^a A single dose of 25 mg. of niacin was given orally on the 65th day.

TABLE III
Hematologic Data on 8 Low-Protein Control Animals

Pig	Days on exp.	RBC mill./mm. ³	Hgb g-%	Ht ml./100 ml.	MCV μ^3	MCH $\gamma\gamma$	MCHC per cent	Reties per cent	WBC $\times 1000$ mm. ³	I.I.
9-61	115	5.56	11.1	35.0	63	20	32	0.4	14.2	5
9-62	125	6.12	12.5	40.0	65	20	31	1.0	15.1	2
9-63	71	6.20	11.4	35.5	57	18	32	1.2	14.4	3
10-45	132	6.89	13.8	40.0	58	20	34	0.8	15.6	2
10-46	132	6.83	13.1	39.4	57	19	33	2.4	15.6	2
10-47	132	6.96	11.3	34.0	49	16	33	2.0	12.7	2
10-48	132	6.43	12.6	38.0	59	20	33	3.6	17.3	2
10-50	132	6.62	12.3	37.4	56	19	33	1.4	14.7	2

Ht—volume of packed red cells.

MCV—mean corpuscular volume.

MCH—mean corpuscular hemoglobin.

MCHC—mean corpuscular hemoglobin concentration.

I.I.—icterus index.

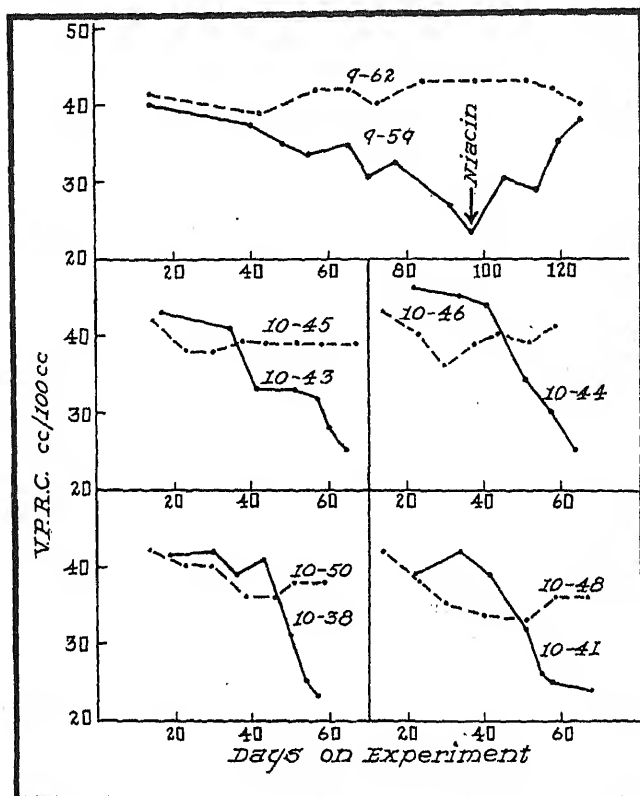


FIG. 1. Development of anemia in 5 animals fed a low-protein, niacin-deficient diet (continuous line). For comparison, the volume of packed red cells of 5 pigs fed the same diet but supplemented with niacin is indicated by the interrupted lines. The response of pig 9-59 to the daily intramuscular injection of 12 mg. of niacin/kg. of body weight is also shown.

thiamine hydrochloride, 0.25; riboflavin, 0.12; nicotinic acid, 1.20; pyridoxine hydrochloride, 0.20; calcium pantothenate, 0.50; choline chloride, 10.0; inositol, 0.10; *p*-aminobenzoic acid, 0.10; pteroylglutamic acid, 0.30. Biotin, when administered, was given intramuscularly, 50 γ /kg. of body weight per week. The experimental details are summarized in Table I. No difference was noted in the effects produced by the high-fat and the high-carbohydrate diets. Full details of the experimental methods have been published elsewhere (9).

RESULTS

The hematologic data on 12 animals fed a niacin-deficient, low-protein diet are presented in Table II. In each instance anemia developed. Significant anemia was

generally present within 50 days after the experiment was started. Maximal anemia developed between 60 and 120 days and was frequently associated with a slight reticulocytosis. With one exception, the anemia in each was normocytic and normochromic. In one animal (10-37) the mean corpuscular volume was $70 \mu^3$. It may be significant that this animal received no pteroylglutamic acid but it should be noted that anemia developed whether or not pteroylglutamic acid was added to the diet, and no difference in the severity of the anemia was noted. In no instance was leukopenia observed nor was there a thrombocytopenia.

Hematologic data for 8 animals receiving a low-protein diet supplemented with 1.20 mg. of nicotinic acid/kg. of body weight daily are presented in Table III. Al-

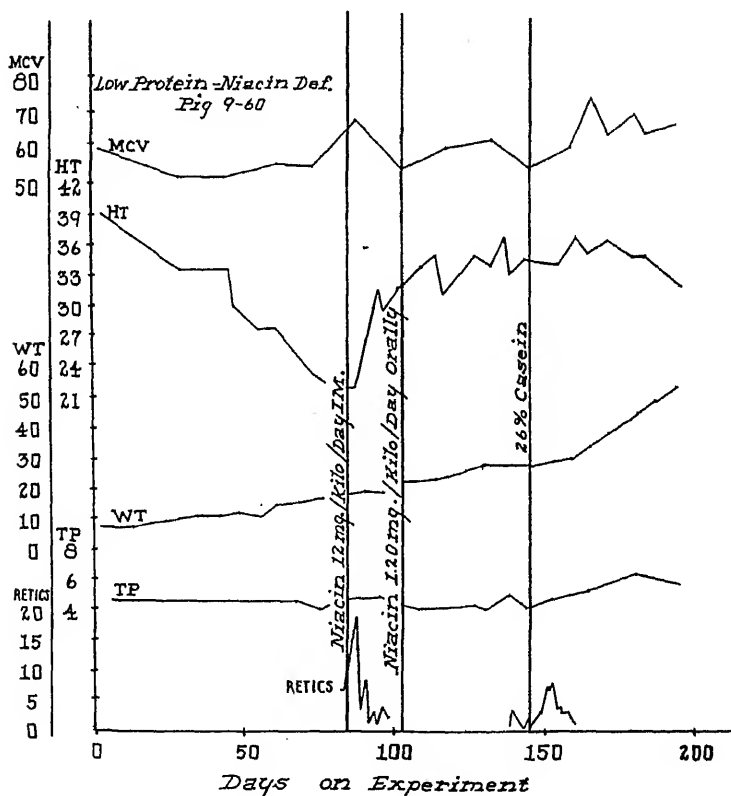


FIG. 2. Development of anemia in a pig (9-60) fed a low-protein diet not supplemented with niacin. The response to niacin and the changes occurring when the level of casein was raised from 10% to 26% are also shown.

MCV refers to mean corpuscular volume in μ^3 , Ht. refers to volume of packed red cells in ml./100 ml., T. P. refers to total serum proteins in g.-%. Reticulocytes are expressed in per cent. Weight is expressed in kg.

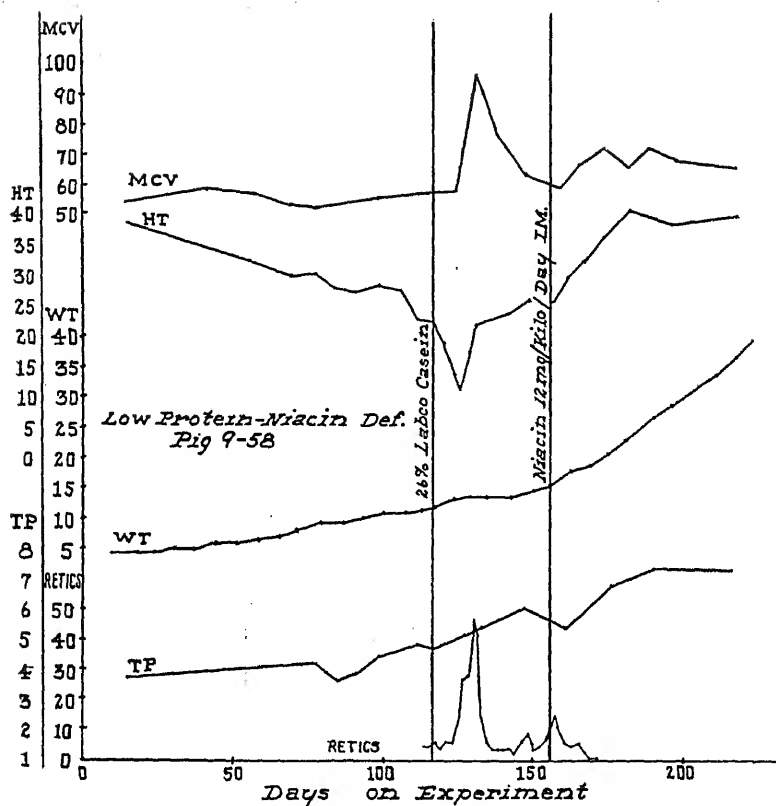


FIG. 3. Development of anemia in a pig fed a low-protein diet not supplemented with niacin. A reticulocyte response and a moderate rise in volume of packed red cells followed the administration of "vitamin-free" casein (Labco) at a level of 26%. Further relief of anemia occurred when niacin was given. For symbols see Fig. 2.

though slight anemia was present after 70-132 days, the anemia was not comparable in degree to that seen in the niacin-deficient group. In Fig. 1 the changes in the volume of packed red cells of 5 niacin-deficient animals are compared with those of 5 controls. As can be seen, the decline in the volume of packed red cells was considerably greater in the niacin-deficient than in the control animals. The response of one animal (9-59) to the daily intramuscular injection of 12 mg. of niacin/kg. of body weight is also shown in Fig. 1.

The response of another pig (9-60) fed a low-protein, niacin-deficient diet to the administration of niacin is shown in detail in Fig. 2. When the volume of packed red cells had fallen to 21 ml./100 ml. blood, niacin was administered intramuscularly in a dosage of 12 mg./kg. of body weight daily for 20 days. A reticulocytosis of 19.6%

followed immediately and the volume of packed red cells rose. The animal was then maintained on 1.2 mg./kg./day of niacin. Sixty days after niacin therapy was started the casein was increased to 26%. A slight reticulocytosis occurred and there was a marked increase in growth but no further rise in the volume of packed red cells took place.

The response of a pig (9-58) fed a low-protein, niacin-deficient diet to the administration of protein is shown in Fig. 3. Sixteen days after the casein had been increased from 10% to 26% the reticulocytes reached a maximum of 47.0%. This was followed by a rise in the volume of packed red cells from 11.0 ml. to 25.5 ml./100 ml. blood. On the 155th day of the experiment, niacin, 12 mg./kg./day, was given intramuscu-

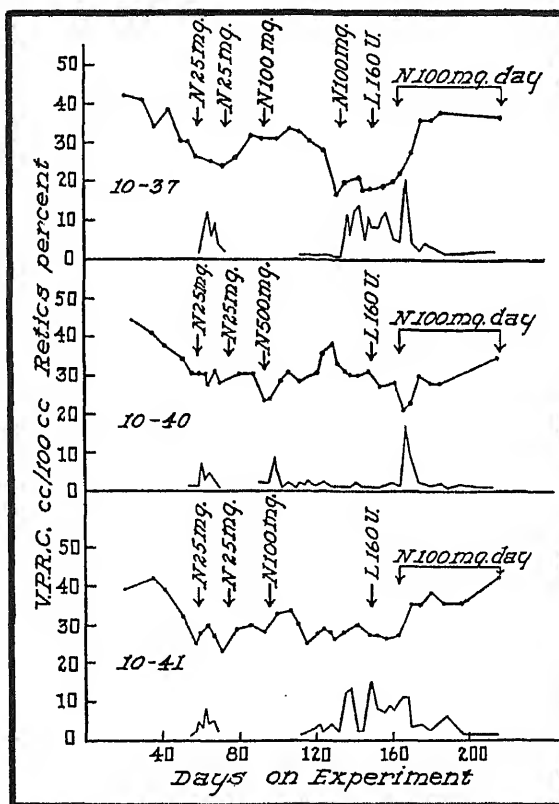


FIG. 4. Effect of the intramuscular injection of 160 units of liver extract (Lederle, 15 units/ml.) in 3 animals maintained on a low-protein, niacin-deficient diet after niacin had been given in various single doses several times. Note the response to large oral doses of niacin after the negative effects of liver extract. Legends same as in Fig. 2.

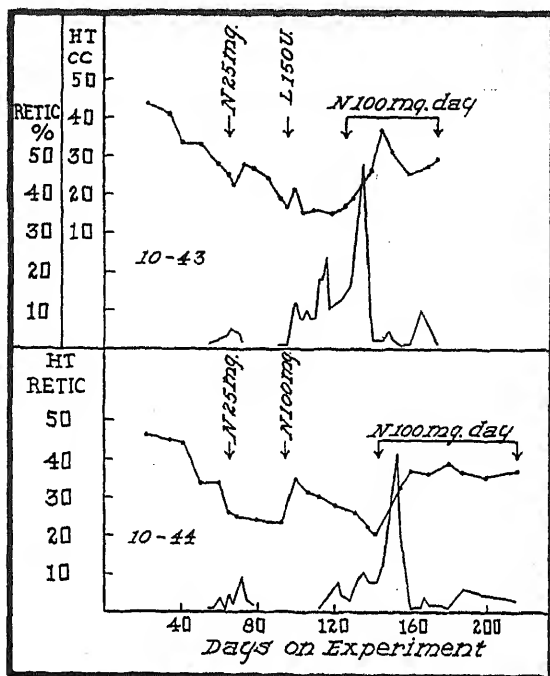


Fig. 5. Similar observations in 2 more pigs. Note the response to large oral doses of niacin in an animal (10-44) which had not previously received liver extract.

larly. This was followed by a moderate reticulocytosis (16%) and a further rise in the volume of packed red cells to 40 ml./100 ml.

To determine whether animals repeatedly depleted of niacin would eventually fail to respond to niacin and exhibit a deficiency of a factor contained in highly purified liver extract, 5 animals were given small doses of niacin at intervals throughout a 216-day experiment. The results are presented in Figs. 4 and 5. An initial dose of 25 mg. of niacin was given orally on about the 60th day of the experiment. All 5 animals exhibited a slight reticulocytosis, but this was not associated with a significant rise in the volume of packed red cells although the rapid fall was delayed. The administration of further small doses of niacin (25-100 mg.) either temporarily delayed the development of further anemia or resulted in a significant but unsustained rise in the volume of packed red cells. Four of the 5 animals were eventually treated with purified liver extract (Lederle, 15 units/ml.) intramuscularly. One animal (10-37) had received no pteroylglutamic acid prior to liver therapy. In none of the animals was there a significant rise in the volume of packed red cells following liver therapy. In one animal (10-43) an irregular reticulocytosis appeared after the liver therapy. However, there was no concomitant rise in volume of packed red cells and, since,

spontaneous reticulocytosis did occur in other animals (10-41, 10-44), no significance can be attached to this. After the 4 animals failed to respond to liver extract, they were treated with 100 mg. of niacin daily by mouth. In 3 of the animals (10-37, 10-40, 10-43) not only did reticulocytosis follow but in all 4 of the animals there was a rise in the volume of packed red cells to a level equivalent to that of the control animals maintained on a low-protein diet for a comparable period. To determine whether a response to niacin would occur in an animal not previously treated with liver, one (10-44) was given no liver extract. A significant and rapid response of both reticulocytes and volume of packed red cells followed the administration of 100 mg. of niacin daily.

DISCUSSION

The data presented indicate that niacin is essential for normal erythropoiesis in pigs fed a diet low in protein. Lack of niacin is associated with the development of a moderately severe, normocytic anemia. This anemia could be prevented from developing, and, when present, could be relieved, by the administration of niacin.

Determinations of erythrocyte protoporphyrin and plasma iron were made in several of the animals at the height of the anemia and prior to therapy. No significant alteration from the normal was found.

Recent studies have shown that tryptophan can be utilized for the synthesis of nicotinic acid (10). In view of this, the beneficial effect, in pigs 9-58, of a diet containing "vitamin free" casein (Labco) at a level of 26% is not surprising, since the intake of tryptophan was thereby increased more than 2-fold. At this level of protein intake niacin deficiency does not develop in the pig (8).

It should be pointed out that the administration of either niacin or a diet containing 26% casein in pigs 9-58 and 9-60 did not result in a significant increase in growth even though the anemia was relieved. Only when both were added did a good growth response occur.

The studies reported here do not refute those published by the Wisconsin group (6, 7), since the conditions of the two experiments were quite different. Elvehjem and his co-workers used diets containing 30, 24, or 19% casein and fed these to dogs. Furthermore, the casein used by them was highly purified whereas the crude casein used in these experiments has been shown to contain significant amounts of "extrinsic factor" (11).

A number of the animals in this study developed marked ataxia during the progress of the experiment. This will be the subject of a separate report.

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We are indebted to Mrs. Darlene Kehl, Mr. George Trappett and Mr. Ocie Hadley for technical assistance.

SUMMARY

1. Swine maintained on a low-protein, niacin-deficient diet developed a normocytic anemia which responded to either protein or niacin therapy.

2. Swine maintained on a low-protein diet with added niacin failed to develop a significant degree of anemia in a comparable period of time.

3. Repeated niacin depletion over a period of 216 days did not result in the production of a demonstrable deficiency of the antipernicious anemia liver substance under the conditions of these experiments.

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Inhibition of Enzymatic Proteolysis. I. Observations with Carbonyl Group Reagents; Effect of Hydrazine on Peptic Hydrolysis¹

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INTRODUCTION

Rona investigated in 1920 (1) the effect of several carbonyl group reagents on pepsin and trypsin and found no decrease in proteolytic activity. The reagents used were: sodium bisulfite, hydroxylamine, potassium cyanide, phenylhydrazine, and benzene sulphydroxamic acid. Dernby (2) reported inhibition of yeast peptidases by cyanide. Josephson and Euler (3) observed inhibition of "erepsin" (a mixture of peptidases from hog intestine) by phenylhydrazine, cyanide, and bisulfite and speculated that this intestinal peptidase contains a group of aldehyde character. Waldschmidt-Leitz and Rauchalles (4) agreed with this assumption when they found that the pH-curve of the condensation velocity of glycylglycine with glucose coincided with the pH-activity curve of intestinal dipeptidase acting on the same substrate. The conclusion was drawn that the peptide reacted in both cases with chemically similar groups and that, therefore, "erepsin" contained an aldehyde group, as suggested by Josephson and Euler.

These reports attracted little attention, however, and later investigators expressed the view that proteolytic enzymes contain no constituents other than the usual L-amino acids in peptide linkages.

Gailey and Johnson (5) studied the activation of erepsin dipeptidases by metals and found that one of the enzymes contained in the mixture hydrolyzed glycylglycine and was strongly activated by cobalt. On the basis of this report, one might be inclined to assume that the cyanide inhibition described by Josephson and Euler was the result of a complex formation between inhibitor and activating metal ions. Inhibition of trypsin by cyanide, hydrogen sulfide, cysteine, and pyrophosphate was reported by Grassmann, Dyckerhoff and von Schoenebeck (6). The original explanation, that this inhibition was due to an inactivation of trypsin kinase, had to be revised when Grob (7) observed inhibition of crystalline trypsin (which required no kinase) on addition of reducing agents.

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There are numerous reports describing activation of proteolytic enzymes by cyanide and a variety of reducing agents. Papain, cathepsin II, III, and IV, for example, are activated by reducing agents and are accompanied in plant and animal tissues by natural activators in the form of sulfhydryl compounds (6). Various suggestions have been made for the mode of action of these substances. Krebs (8) felt that the activation by cyanide was due to the removal of interfering metal ions through complex formation. Bersin (9) postulated that these effectors acted by reducing disulfide linkages in the enzyme molecule to sulfhydryl groups and that this resulted in an activation if only the SH-form of the particular enzyme was catalytically active. Inhibition of certain enzymes by reducing agents might then be the result of the disruption of essential disulfide linkages. Bergmann and Fruton (10, 11) questioned the validity of the sulfhydryl or oxidation-reduction theory. Fruton and Bergmann (12) showed, in the case of papain, that the activation by cyanide was due (in part at least) to the combination of the activator with the proteinase in a coenzyme-like manner. Earlier findings by Bergmann and Ross (13, 14) might perhaps be explained by the presence of an aldehyde group in papain, but conclusive evidence to substantiate this possibility was not obtained. It is quite likely that at least 3 mechanisms (reaction with metals, reduction of disulfide linkages, and association with the enzyme) play a role in the mode of action of these reducing agents, individually or together, depending on the particular enzyme.

The complexity of the situation may be illustrated by results obtained by Maschmann in studies on the role of metal ions as activators for peptidases. Maschmann (15) observed that serum peptidases were activated by manganese ions and inhibited by cyanide and cysteine. The effect of cyanide persisted on addition of manganese; the inhibition by cysteine was reversed when small amounts of $MnSO_4$ were added. This eliminated the possibility that cysteine inhibited by reducing disulfide linkages. Aldehyde reagents did not inhibit these enzymes, except in a few unexplained instances with phenylhydrazine, so that the presence of carbonyl groups (which could have reacted with cyanide and cysteine) was considered very unlikely.

It seemed desirable, in view of the many unsolved problems, to reinvestigate the possibility of the existence of additional groups in proteolytic enzymes, *i.e.*, of groups which are not part of the usual amino acids. This report describes preliminary observations on the effect of carbonyl reagents on pepsin, trypsin, and papain, and a more detailed investigation of the inhibition of peptic activity by hydrazine.

EXPERIMENTAL

1. *Enzymes*

The enzyme preparations used in the following experiments were: papain (Merck); trypsin 1:300 (Nutritional Biochemical Corporation); pepsin, granular, 1:10,000 (Pfanstiehl); and crystalline pepsin (porcine origin, Armour and Co.). On a weight basis, the cryst. pepsin was 4-5 times as active against egg-white as the crude, granu-

lar material obtained from Pfanstiehl. The commercial cryst. product was found to contain 14.1% nitrogen. (Kjeldahl method, corrected for 8.4% moisture).

2. Substrates

a. *Egg-White Suspension.* This substrate, which was used in the majority of the experiments described here, was prepared according to Riggs and Stadie (16), except that a Waring Blendor was substituted for the homogenizer used in the original procedure. The stock substrate (egg-white blended with 10 ml. water/g.) was diluted 5-fold with water for the preliminary experiments (macro method), and with an equal volume of water for subsequent experiments (micro method).

b. *Casein.* Five g. Labco casein were moistened with some water, 40 ml. 0.1 N NaOH was added, and the mixture left in the refrigerator overnight. The volume was then brought to 500 ml. by addition of water, and the solution filtered. Before each test, the acidified substrate was prepared by adding 2 volumes 0.1 N HCl to 3 volumes casein stock solution.

c. *Albumin.* This substrate was composed of equal volumes of 0.1 N HCl and a 1% solution of bovine albumin (fraction V, Armour and Co.) in 1% NaCl solution. The mixture was prepared fresh before each experiment.

d. *Carbobenzoxy-L-glutamyl-L-tyrosine.* This material was synthesized as described by Bergmann and coworkers (17). It was used in a concentration of 0.05 millimole/ml. incubation mixture.

3. Preliminary Experiments

The following mixtures were incubated in colorimeter tubes (18.5 mm. diameter) at 30°C.: 5 ml. egg-white substrate, 4 ml. buffer, 1 ml. enzyme solution, and 1 ml. water or inhibitor solution. Blank tubes contained water instead of enzyme. When necessary, additional blank tubes for enzyme color and turbidity and for inhibitor color were also incubated (water was substituted for substrate) and corrections made for the light absorption of these solutions. The enzyme solutions were: 0.01% crude pepsin solution, 0.1% papain solution (filtered), and 1% trypsin solution (filtered). The incubations with trypsin and papain were carried out at pH 7.0 (*m*/15 phosphate buffer) and with pepsin at pH 1.50 (*m*/5 citrate buffer). Inhibitor solutions were brought to the pH of the incubation mixtures before addition.

The decrease in turbidity was followed photoelectrically, using a Lumetron colorimeter and filter M-420. The concentration of residual substrate at various periods of time was estimated from the extinction measurements with the aid of calibration curves. These curves were obtained by plotting corresponding percentage values of serial dilutions of substrate against extinction.

These preliminary tests revealed that a variety of carbonyl group reagents interfered with the enzymatic processes. Representative results are shown in Table I.

The figures for "per cent hydrolysis" were obtained as described in the experimental part. To avoid confusion it should be kept in mind that 27% hydrolysis (for example) is meant to convey the observation that the turbidity of the incubation mixture was identical with that of a mixture of 73 parts of undigested substrate (buffer added and water substituted for enzyme) with 27 parts of appropriately diluted buffer. The

TABLE I
*Effect of Aldehyde Reagents on the Proteolysis of
 Egg-White by Pepsin, Trypsin, and Papain*

Inhibitor	Pepsin				Trypsin				Papain		
	Per cent hydrolysis				Per cent hydrolysis				Per cent hydrolysis		
	5 min.	10 min.	20 min.	30 min.	5 min.	10 min.	20 min.	30 min.	1 hr.	2 hr.	3 hr.
(mole/l.) None	27	45	63	76	36	48	61	69	26	31	34
NaHSO ₃ (0.008)	22	36	52	64	Activation				Activation		
Dimedon (0.007)	20	36	58	70	No inhibition				No inhibition		
NH ₂ NHCONH ₂ (0.012)	19	36	54	65	30	42	54	61	19	24	26
C ₆ H ₅ NHNH ₂ (0.009)	18	32	54	67	28	38	44	54	14	18	20
NH ₂ OH (0.026)	16	29	50	62	28	36	45	52	17	20	22
NH ₂ NH ₂ (0.02)	8	13	21	26	18	30	41	49	13	18	21

figures refer only to the breakdown of the egg-white into soluble peptides and do not take into consideration any further hydrolysis which might take place after the fragments have ceased to cause turbidity.

4. Inhibition of Peptic Hydrolysis of Egg-White by Hydrazine

The inhibitory effect of hydrazine on peptic hydrolysis was selected for further study. The composition of the incubation mixture was as described above. Portions of these mixtures were transferred to micro colorimeter tubes (6.5 mm. diameter) and a galvanometer with 10 times the sensitivity of the regular instrument was used as zero point indicator. The incubation took place at room temperature, which was measured with a thermometer kept in the micro tube compartment of the colorimeter. Each experiment consisted of two sets, one with and one without inhibitor, both at pH 1.50. The start of the reaction was timed accurately as the moment the pepsin solution was added to the substrate-buffer and substrate-buffer-inhibitor mixture,

respectively. Transmission readings were taken at 30 sec. intervals for a period of 15 min., converted to extinctions and evaluated (as described above) with the aid of calibration curves. The turbidity of blank tubes, in which water took the place of pepsin solution, was determined at 5 min. intervals. No noticeable changes occurred in these mixtures. The extinction of these blanks represented that of the initial substrate concentration. Similar to the experience of Riggs and Stadie (16), a straight line was obtained for the time interval from 300 to 600 sec., when $\log \frac{a}{a-x}$ was plotted against time (a is the initial substrate concentration, $a-x$ is the substrate concentration at time t , obtained by subtracting the per cent hydrolysis² observed at time t from the initial substrate concentration, i.e., from 100%).

This linear relationship permitted the determination of a first order constant for each reaction (see Fig. 1). This constant, k , valid for the

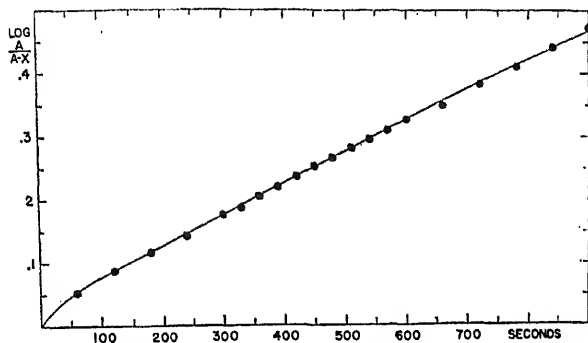


Fig. 1. Graphical determination of first order reaction constant.

Egg-white substrate; crude pepsin, concentration 4.5 mg./100 ml. incubation mixture; a = substrate concentration at time zero; $a-x$ = substrate concentration at time t . The reaction constant k is calculated from the slope of the linear portion of the curve.

period from 300 to 600 sec., was taken as the slope of the linear portion of the curve. Values of k were determined for concentrations of crude pepsin from 1 to 10 mg./100 ml. incubation mixture. A straight line was obtained when these k values were plotted against pepsin concentrations (see Fig. 2), indicating that k was a direct measurement of the amount of active pepsin in the mixture. All subsequent experiments with egg-white were interpreted on the basis of the numerical value of this reaction constant. *No difference was observed in any of the results to be described, when enzymatically equivalent amounts of crystal-*

² See remarks under Table I.

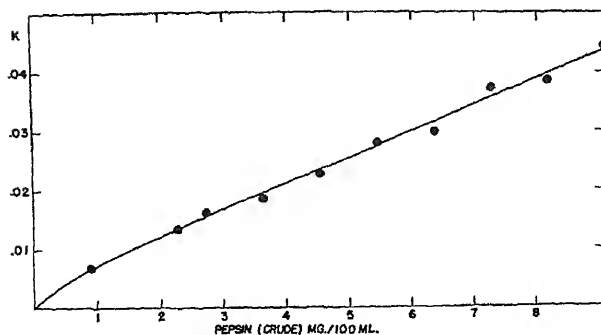


FIG. 2. Linear relationship between first order reaction constant and pepsin concentration.

line pepsin were used instead of crude pepsin. The following results were obtained: (a) Two millimoles hydrazine/l. incubation mixture decreased k to one-half its original value, *i.e.*, produced 50% inhibition.

There was no increase in the degree of inhibition when a mixture of pepsin and hydrazine was kept for 50 hrs. at 4°C. (at pH 4.1 and pH 6.2) prior to the incubation

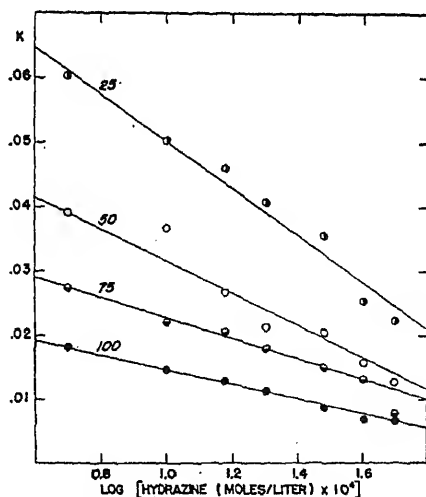


FIG. 3. Effect of hydrazine concentration on peptic activity.

The numbers on the curves refer to the relative initial substrate concentrations in per cent of the usual concentration, which is arbitrarily labeled 100. Pepsin concentration: 4.5 mg./100 ml.

with egg-white. Similarly, the addition of hydrazine to the substrate 3 days before the incubation experiment (and storage during this period at 4°C. and pH 7.1) did not alter the degree of inhibition.

(b) There was a linear relationship between the observed values of k and the logarithms of the hydrazine concentrations (see Fig. 3). (c) Both in the presence and absence of hydrazine, there was a linear relationship between k and the reciprocal of the initial substrate concentration, as shown in Fig. 4. (d) The customary graphical interpreta-

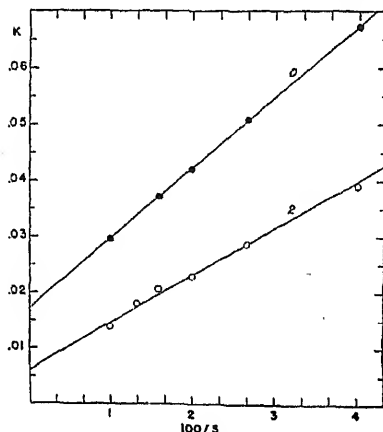


FIG. 4. Effect of substrate concentration on peptic activity.

Pepsin concentration: 4.5 mg./100 ml. Curve 0: no inhibitor, Curve 2: in presence of 2 millimoles hydrazine/l. incubation mixture.

tion of experimental data indicated that the inhibition was of the non-competitive type, as illustrated in Fig. 5.

(e) As a by-product of numerous tests without inhibitor, it was found that a linear relationship existed between the reciprocal of the absolute temperature at which the experiments were performed (293–303° K.) and $\log k$. The apparent activation energy was determined from this relationship as 19,250 cal./mole, which is in reasonable agreement with a literature value of 17,700 cal./mole for casein as substrate and temperatures from 1 to 30°C. (18). The temperature coefficient (20–30°C.) was determined as 2.98.

5. Other Inhibitors for the Peptic Hydrolysis of Egg-White

Results with 3 additional carbonyl group reagents, obtained in the same manner as described for hydrazine, were as follows: *Dimedon*, 0.2

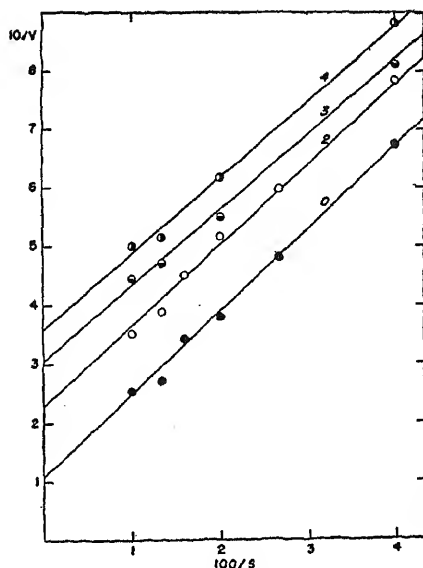


FIG. 5. Graphical presentation of data indicating that the inhibition of pepsin by hydrazine is noncompetitive.

S = initial substrate concentrations in relative numbers; V = overall velocity for a period of 15 mins. The numbers on the curves refer to hydrazine concentrations in millimoles/l.

millimole/l.: no inhibition; 20 millimoles/l.: 23% inhibition. *Sodium bisulfite*, 2 millimoles/l.: 8% inhibition; 20 millimoles/l.: 48% inhibition. *Girard's Reagent T*, 20 millimoles/l.: 15% inhibition; 100 millimoles/l.: 52% inhibition; 180 millimoles/l.: 76% inhibition.

The conclusions in regard to the effect of carbonyl group reagents on the proteolysis of egg-white are based on nephelometric data. With the equipment used, these data were highly reproducible and the inhibitions shown in Table I were far greater than the variations from one experiment to the next. Furthermore, each claim for inhibition is based on at least 6 independent runs and each time enzyme with and without inhibitor was tested simultaneously. However, it seemed conceivable that the inhibition of the proteolysis of egg-white might be simulated by an effect of the inhibitor on the colloidal state of the substrate. Experiments with additional substrates using different methods for the measurement of enzymatic activity seemed therefore indicated.

6. *Effect of Hydrazine on the Peptic Hydrolysis of Beef Albumin, Casein, and Carbobenzoxy-L-glutamyl-L-tyrosine*

a. Albumin. The incubation mixtures consisted of 5 ml. acidified substrate, 1 ml. water or hydrazine solution, and 1 ml. pepsin solution, containing either 0.01 or 0.02 mg. cryst. pepsin. In all incubation mixtures the pH was kept at 1.67 ± 0.05 (glass electrode). The hydrolysis took place at 37.5°C . At specified times, 4 ml. of 10% trichloroacetic acid solution was added to stop the reaction and to precipitate proteins. The mixtures were filtered and the extinction of each filtrate was measured at $275\text{ m}\mu$ (absorption maximum of tyrosine and tryptophan) with a Beckman spectrophotometer against filtrates from blank tubes. The latter contained water instead of pepsin solution and had been processed in the same way as the experimental tubes, including incubation for accurately measured periods of time. The light absorption of the substrate at $275\text{ m}\mu$ was found to be the same (corrected for dilution) as that of a protein-free filtrate obtained after complete peptic digestion of the substrate. The extinction of the substrate was therefore used as the numerical value for the concentration (a) of the substrate at time zero. The extinction of the filtrates gave numerical values for the amount of substrate (x) which had been hydrolyzed to trichloroacetic acid-soluble fragments up to the moment the experiment was stopped. It is most likely that these filtrates still contained polypeptides which would have been hydrolyzed further on prolonged contact with pepsin.

The kinetic evaluation of these measurements is, therefore, to be considered only as a crude approximation. Despite this remark it must be kept in mind, however, that the measurement of free and combined tyrosine and tryptophan in trichloroacetic acid filtrates is considered

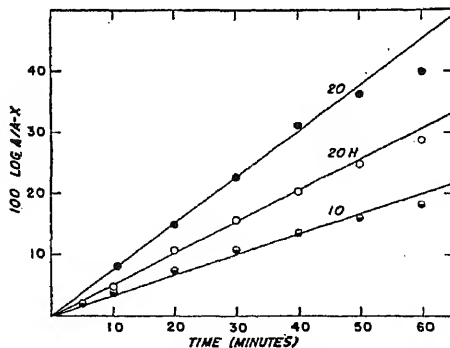


FIG. 6. Graphical determination of first order reaction constant.

Beef albumin substrate; cryst. pepsin concentration: 0.010 mg./7 ml. in curve 10, 0.020 mg./7 ml. in curves 20 and 20 H. Curve 20 H: 100 millimoles hydrazine present /l. incubation mixture. The reaction constant is calculated from the slope of the lines.

at present the most satisfactory method for the determination of peptic activity (19). When $\log (a/a-x)$ was plotted against time, straight lines resulted, as shown in Fig. 6. The first order reaction constant k was determined from the slopes of these lines. A comparison of the curves for 10 and 20 γ pepsin (Fig. 6) shows a reasonable relationship between k (slopes) and enzyme concentration, indicating that the objections mentioned above do not seriously interfere with the usefulness of this evaluation procedure. The presence of 100 millimoles hydrazine/l. incubation mixture resulted in 32% inhibition, i.e., k was 68% of that found in absence of hydrazine.

b. *Casein*. The experiments were performed and evaluated exactly as described for albumin. The pH of all incubation mixtures was kept at 1.76–1.78 (glass electrode). First order reaction constants were calculated from the ultraviolet absorption measurements. The kinetic evaluation of these measurements is, of course, subject to the same objections as mentioned above in connection with albumin. The speed of formation of trichloroacetic acid-soluble fragments, measured by the appearance of free and combined tyrosine and tryptophan in the filtrates, decreased more rapidly during the course of each experiment than expected for a first order reaction. This does not necessarily mean that the speed of hydrolysis is decreased, as our measurements do not reveal the further splitting of polypeptides after they have become soluble in trichloroacetic acid. The average values for k listed in Table II are the arithmetic means of 6 values obtained at 10 min. intervals.

TABLE II
Effect of Hydrazine on the Hydrolysis of Casein by Crystalline Pepsin
(Temp. 37.5°C.; Casein: 0.43 g./100 ml.; pH: 1.76–1.78)

Pepsin	Hydrazine	$k \times 10^4$ ^a			Inhibition
		10 min.	60 min.	Average	
<i>mg./7 ml.</i>	<i>mole/l.</i>				<i>per cent</i>
0.010	0	46	39	44	—
0.010	0.01	32	27	30	33
0.010	0.02	27	19	22	50
0.020	0	96	59	80	—
0.020	0.01	56	43	51	37

$$^a k = \frac{1}{t} \log \frac{a}{a-x}$$

The presence of 20 millimoles hydrazine/l. reduced the average k values to $\frac{1}{2}$ of the values observed in absence of inhibitor. Similarly reduced values for k were obtained when only half as much enzyme was used in absence of hydrazine. This observation justifies to some extent the calculation of "per cent inhibition" from the ratio of k values in presence and absence of inhibitor.

c. Carbobenzoxy-L-glutamyl-L-tyrosine. The incubations with this compound were carried out at 37.5°C. and pH 4.0 (glass electrode) in $m/5$ citrate buffer. The progress of the hydrolysis was followed with the titrimetric ninhydrin method as described by Van Slyke *et al.* (20). Nine millimoles of hydrazine/l. incubation mixture produced 23% inhibition when the enzyme concentration was 7.3 mg. cryst. pepsin/ml., and 13% inhibition when the pepsin concentration was increased to 20 mg./ml. Monomolecular reaction constants were used for the calculation of the inhibition percentages.

DISCUSSION

The results of the preliminary experiments (Table I) and of the more detailed investigation of the effect of hydrazine on peptic hydrolysis, show that the proteolysis of a variety of substrates was slowed down considerably when carbonyl group reagents were added to the incubation mixtures. Exceptions occurred with bisulfite, which activated trypsin and papain, and with dimedon, which had no effect on the activity of these two enzymes. Dimedon inhibited peptic hydrolysis, and its failure to interfere with the activity of trypsin and papain is perhaps due to an insufficient concentration of this compound.

The observations with trypsin and papain are considered preliminary, as only crude enzymes were available for this work. The results with pepsin, however, were identical whether crude or crystalline enzyme was used.

It is not possible to explain at the present stage of this work the mechanism by which carbonyl group reagents interfere with peptic activity. It seems permissible, nevertheless, to discuss a few of the many possibilities for the mode of action of these inhibitors.

First of all, there are two alternatives: the inhibitors react either with the substrates or with the enzyme.

A reaction of carbonyl group reagents with the substrates is quite possible. Various observations favor such an assumption. For example: the concentration of hydrazine needed for 50% inhibition of peptic activity is different for each substrate. This could be due to differences in the ease with which the various substrates undergo changes, making

them more resistant towards peptic hydrolysis. Furthermore, a reaction between proteins and hydrazines has actually been described by Cheesman and Ehrensvärd (21), who found that the presence of dinitrophenylhydrazine during slow acid hydrolysis of several proteins led to high molecular colored derivatives. The authors consider the possibility that dinitrophenylhydrazine might react with amidine groups in the protein in the same manner as several hydrazines react with simple amidines (such as benzoylaminoacetamide) under exchange of the imino group against the reactive group of the hydrazines. The existence of amidine groups in proteins is hypothetical, but they could be formed in the condensation of two peptide linkages, one being in the enol form.

Regular peptide linkages are apparently not reacting with hydrazine. Heating of simple acid amides with hydrazine leads to the formation of acid hydrazides (22) through displacement of the more volatile ammonia. Substituted amides, however, such as the dipeptides glycylglycine and leucylglycine were found to be inert against treatment with dinitrophenylhydrazine (21). This is significant in view of our results with carbobenzoxyglutamyltyrosine and speaks against the possibility that the inhibition of the peptic hydrolysis of this peptide was due to its reaction with hydrazine. However, this evidence is not absolute proof, and experiments are, therefore, in progress to test analytically whether a reaction occurs between this peptide and hydrazine derivatives.

Turning to the other alternative, namely, that the inhibitions described in this report might be due to a reaction between pepsin and the carbonyl group reagents, one must keep in mind that pepsin is a protein. All possibilities for the reactions of carbonyl group reagents with proteins are, therefore, also possibilities for a reaction with pepsin. This includes reactions with hypothetical amidine groups as well as such nonspecific reactions as, for example, partial denaturation by the inhibitors, or their adsorption on the surface of the protein molecule. A few obvious explanations for the mode of action of these inhibitors can be easily eliminated. Pepsin is not activated by metal ions and a complex formation between inhibitor and activators of this type, therefore, need not be considered. Hydrazine is a reducing agent, but the hydrazine concentration producing 50% inhibition (2 millimoles/l. with egg-white as substrate) is generally regarded as being too low (23) to reduce disulfide linkages. To test the possibility that the

inhibition might be the result of a reduction, experiments were performed using thioglycolic acid as a reducing agent. No inhibition of the reaction between pepsin and egg-white was observed in presence of 20 millimoles thioglycolic acid/l. incubation mixture.

The kinetic data presented in the experimental part are obviously not to be relied upon too heavily, as has been pointed out before. The graphical correlation between reciprocals of substrate concentrations and reciprocals of reaction velocities (Fig. 5) permits, therefore, only tentatively the conclusion that the inhibition seems to be of the non-competitive type. In regard to pepsin, this would mean that the groups reacting with carbonyl reagents would be "activating centers," not directly participating in the process of proteolysis but essential for full enzymatic activity. The hydroxy groups of the tyrosine molecules in pepsin belong in this category, but they are not likely to react with carbonyl group reagents.

Several instances are known where the inhibition of enzymes by carbonyl reagents is due to the presence of an aldehyde group in the prosthetic groups of these enzymes. Pepsin is not known to have a prosthetic group, but this does not eliminate the possibility that it might contain groups of an aldehydic, ketonic, or closely related nature. A definite decision in this direction could be made if it were possible to isolate a pepsin derivative, such as a hydrazone. Experiments aimed at the isolation of a dinitrophenylhydrazone have been started. It is further planned to decide the question whether the inhibitors combine with the proteolytic enzyme or its substrates through the use of colored inhibitors and inhibitors containing isotopes.

SUMMARY

1. The proteolysis of egg-white by pepsin, trypsin and papain was inhibited by small amounts of hydroxylamine, semicarbazide, phenylhydrazine, and hydrazine.
2. The peptic hydrolysis of egg-white was also inhibited by bisulfite, dimedon, and Girard's Reagent T, but not by thioglycolic acid.
3. A study of the inhibition of the peptic digestion of egg-white showed: (a) there was a linear relationship between the first order reaction constants and the logarithms of the hydrazine concentrations; (b) the inhibition seemed to be of the noncompetitive type.

4. Inhibition of peptic hydrolysis by hydrazine was also observed with beef albumin, casein, and carbobenzoxyglutamyltyrosine as substrate.

5. A few possibilities for the mode of action of these inhibitors were discussed, but no definite conclusions could be reached.

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Studies on Lipoxidase. IV. Effect of Changes in Temperature and pH on Lipoxidase Activity as Determined by Spectral Changes in Methyl Linoleate

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INTRODUCTION

Recently it has been shown that a relationship exists between the spectral changes in methyl linoleate and the decolorization of bixin during enzymatic oxidation with lipoxidase. (6) Therefore, either change can be used as a measure of lipoxidase activity. Since the spectral changes in methyl linoleate seem to be a more direct measure of lipoxidase activity than the decolorization of bixin, the effect of temperature and pH on the enzymatic oxidation of methyl linoleate was investigated to determine whether the optimum conditions for these reactions were the same as those previously reported for the coupled oxidation of bixin and soy bean fatty acids. Sumner and Dounce (7) had previously reported an optimum of pH 6.5 and 25°C. for the bleaching of carotene in olive oil in the presence of lipoxidase. These conditions were also confirmed by Sumner and Smith (8) using bixin, soy bean fatty acids, and a lipoxidase preparation purified 100-fold.

The effect of temperature and pH has been reinvestigated using pure methyl linoleate and a lipoxidase preparation that has been purified 110-fold.

EXPERIMENTAL

Reagents

Methyl Linoleate. The methyl linoleate used in these experiments was prepared from soy bean oil by a modification of the Rollett method (4). The soy bean oil was prepared from soy bean meal by ether extraction. The oil was dissolved in ethyl alcohol and saponification carried out at 0°C. The fatty acids were liberated from the soap by

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the addition of 20% sulfuric acid, the temperature remaining below 4°C. during this operation. The fatty acids were collected and washed several times with hot distilled water until all traces of sulfuric acid had been removed. The fatty acids were then dissolved in freshly distilled anhydrous petroleum ether (b.p. 90–100°C.) and anhydrous sodium sulfate added to remove any trace of water that might have been present in the fatty acids. The sodium sulfate was removed by filtration and the liquid cooled to –20°C. Bromine was added slowly with stirring until the red color of the bromine persisted. The solution was allowed to stand at –20°C. for 1 hr., then warmed to 0°C., and filtered. The white tetrabromide was purified by a modification of the method recommended by Kass *et al.* (1). To the ethyl ether solution of the tetrabromide, charcoal was added and the solution shaken for 15 min. The solution was filtered until free of charcoal particles and cooled to –20°C. Petroleum ether (b.p. 90–100°C.) was then added to precipitate the tetrabromide. The tetrabromide was recrystallized 3 times from ethyl ether and petroleum ether. The resulting compound was a white metallic solid, insoluble in petroleum ether and water, soluble in ethyl ether, alcohol and chloroform, and melting sharply at 115°C. Fresh samples of the methyl linoleate were prepared for each series of determinations from the tetrabromide by debrominating with granular zinc in methyl alcohol and esterifying the free acid with 5 *N* methyl alcohol-hydrochloric acid. The resulting ester was fractionally distilled *in vacuo*, and that fraction having an iodine number of 172.0–172.4 used in the oxidation experiments.

Phosphate Buffer. The various phosphate buffers used in these experiments were prepared by mixing 0.05 *M* Na₂HPO₄ with 0.05 *M* KH₂PO₄ until the desired pH value was obtained.

Cyclohexane. The commercial grade of C.P. cyclohexane was purified by using the method suggested by O'Shaughnessy and Rodebush (3), the fraction boiling between 75–80°C. being used in all oxidation studies.

Lipoxidase Solution. A lipoxidase solution which had been purified 110-fold by the method previously described (6) was used for the oxidation of the methyl linoleate.

Procedure

The procedure used in these oxidation studies of methyl linoleate consisted of mixing 0.1 ml. samples of methyl linoleate in ethyl alcohol with 3 ml. of phosphate buffer in test tubes (16 × 150 mm.). The tubes were then placed in a constant temperature bath and allowed to reach the desired temperature. Next, 0.1 ml. of the lipoxidase solution was added and the contents of the tube gently mixed by inverting the tube. The tubes were shaken gently during the runs to assure that sufficient oxygen was present in the digest and that the methyl linoleate was in contact with the enzyme. After 10 mins., 10 ml. of cyclohexane was added to the digest to stop the reaction and extract the methyl linoleate. This was accomplished by shaking the tubes vigorously for 30 sec., allowing the tubes to stand until the 2 layers separated. The top layer of cyclohexane was then removed with a special siphon (5) which filtered the cyclohexane through cotton to remove any traces of water and protein particles that might be present in the layer. The clear solution could then be analyzed by means of the Beckman Spectrophotometer using the ultraviolet attachments.

INFLUENCE OF TEMPERATURE

The influence of increasing the temperature from 20° to 40°C. was investigated at pH values of 6, 7, and 8. At pH 6, no difference in activity could be detected between those determinations made at 20° and those made at 30°C. (see Fig. 1). However, at 40°C. the maximum at

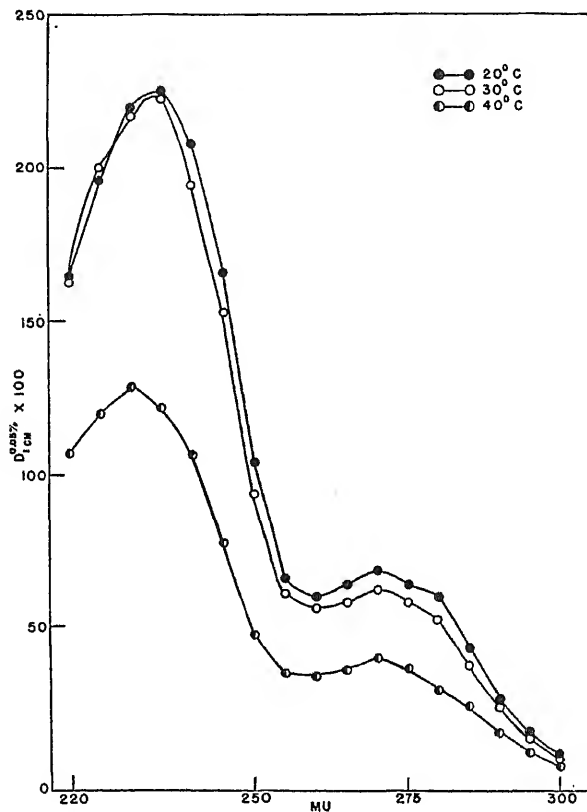


FIG. 1. The spectral changes in methyl linoleate when oxidized at varying temperature at pH 6.

232-235 mμ had attained a value of only 130 ($=D_{1\text{ cm.}}^{0.05\%} \times 100$) as compared with 225 and 223 maxima at pH 6 and 7, respectively. This, therefore, represents a noticeable decrease in activity at 40°C. Increasing the temperature at pH 7, Fig. 2, produced a similar decrease in

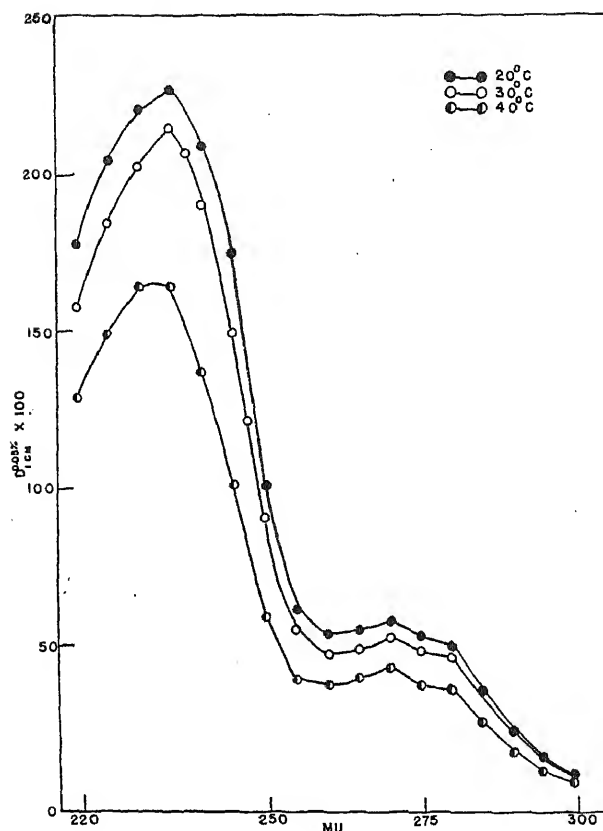


FIG. 2. The spectral changes in methyl linoleate when oxidized at varying temperature at pH 7.

lipoxidase activity at 40°C. There was, furthermore, a slight decrease in activity at 30°C. Both these decreases were, however, slight when compared with the decrease at 40°C. at pH 6.

Fig. 3 indicates a similar decrease in activity with increasing temperatures. Here the decrease was more proportional to each increase in temperature. There was a decrease of 32 units ($= D_{1\text{ cm.}}^{0.05\%} \times 100$) with the first increase of 10° from 20° to 30°C., and an additional decrease of 35 units with an increase in temperature from 30° to 40°C.

The maximum enzymatic activity was attained in all these experiments between 20° and 30°C. Determinations made at temperatures

between 20° and 30°C. indicated that the optimum temperature for soy bean lipoxidase was 25°C.

INFLUENCE OF PH

The influence of changing the pH value of the solution at various temperatures was studied to ascertain the optimum pH value for soy bean lipoxidase activity. Solutions with pH values of 6, 7, and 8 were investigated at several temperatures.

Increasing the pH value from 6 to 8 at 20°C., see Fig. 4, produced

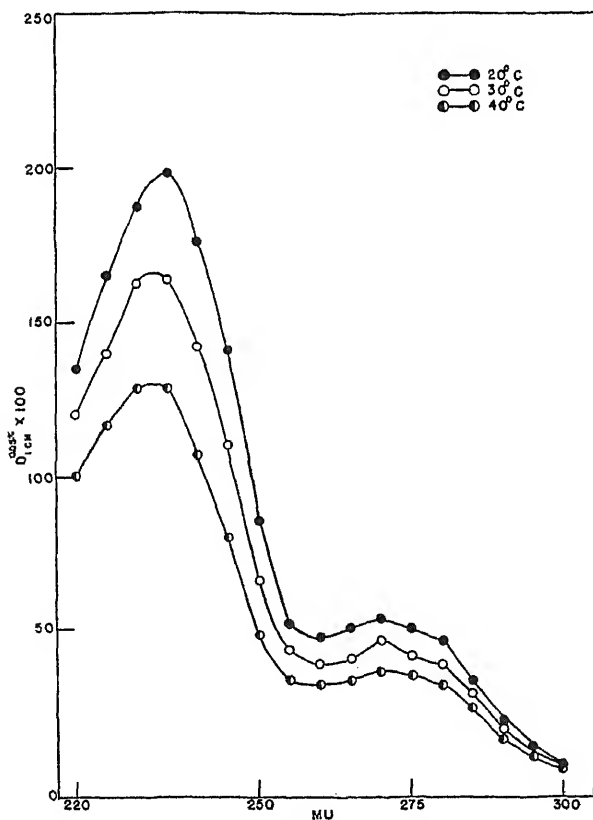


FIG. 3. The spectral changes in methyl linoleate when oxidized at varying temperatures at pH 8.

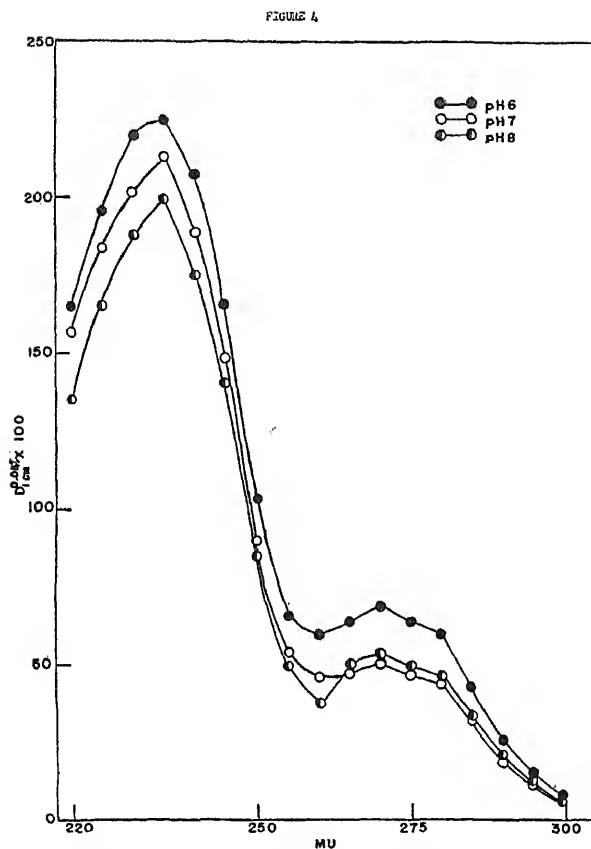


Fig. 4. The spectral changes in methyl linolate when oxidized at varying pH values at 20°C.

only a slight change in activity. At 30°C., however, there was a noticeable decrease in activity at pH 8, see Fig. 5. The maximum in this case being 165 units as compared with maxima of 223 and 227 units at pH 6 and 7, respectively. At 40°C., decreased activity was detectable at all three pH values, see Fig. 6. The activity at pH 7 was, however, not affected to the same extent as the activity at pH 6 or 8. Apparently, at higher temperatures (40°C.) the enzyme is more stable in neutral solutions than in acid or alkaline solutions.

DISCUSSION

From the data presented, the optimum activity of lipoxidase, as determined by spectral changes produced in methyl linoleate during enzymatic oxidation, was attained at pH 6.5 and 25°C. With an increase in temperature above 30°C. there was a decrease in activity at all of the 3 pH values tested, this effect being more noticeable at pH 6 or 8 than at pH 7. At 30°C. maximum activity was obtained at both pH 6 and 7, while a decrease in activity was noticed at pH 8. With temperatures of 20°C. or below, only slight change in lipoxidase activity could be detected with change in the pH value of the solution.

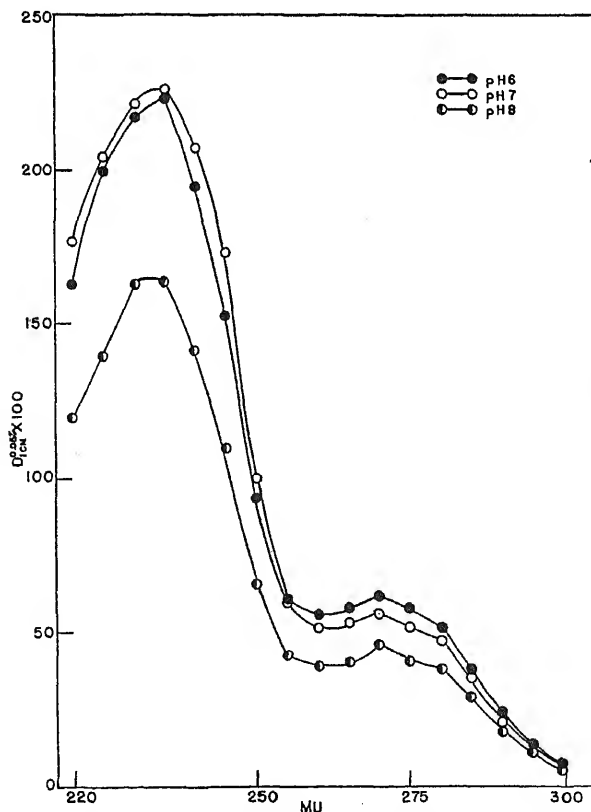


Fig. 5. The spectral changes in methyl linoleate when oxidized at varying pH values at 30°C.

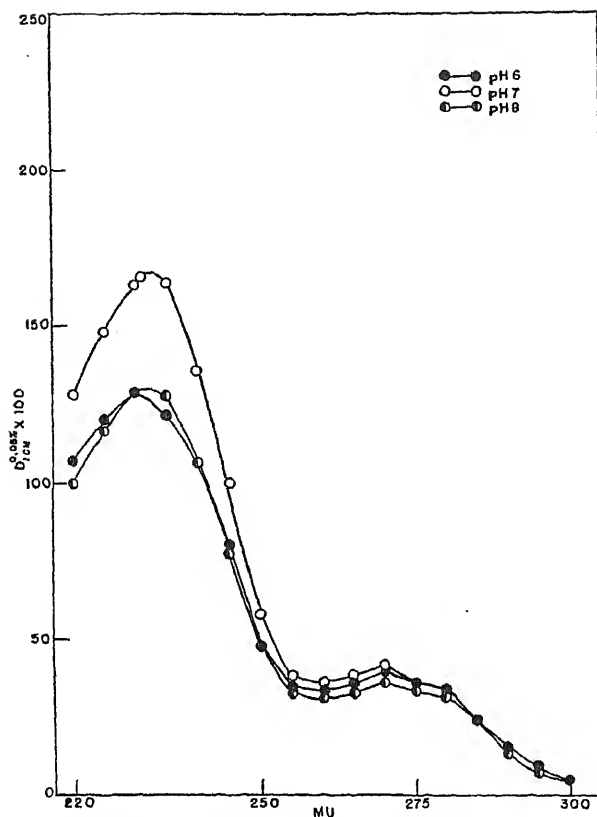


FIG. 6. The spectral changes in methyl linoleate when oxidized at varying pH values at 40°C.

The optimum conditions of pH 6.5 and 25°C. for soy bean lipoxidase activity, as determined by these studies, are in agreement with the data reported by Sumner and Dounce (7) using a mixture of carotene and olive oil in a coupled oxidation with lipoxidase. They are also in agreement with the data of Sumner and Smith (8) using the bixin decolorization method.

Recently, Holman (2) has reported that, "the optimum pH for lipoxidase action lies near pH 9.4." He suggests that this high pH is likely due to the relative inaccessibility of the substrate in the emulsion systems below pH 9, rather than to inactivation of the enzyme below this pH value. If this is the case, Holman has probably measured the effect of pH on the availability of his substrate rather than the effect of pH on his enzyme preparation since no precautions were taken to maintain a constant concentration of available substrate at the various pH values.

If the percentage of light transmitted by a solution of sodium linoleate, as used in a standard determination of lipoxidase activity, is compared at varying pH values, it will be seen that a sigmoidal curve is obtained, see Fig. 7. This curve indicates that maximum transmission of light, or, in other words, maximum solubility of the sodium linoleate exists only at pH 9 or above. Below this value most of the sodium linoleate will exist in a colloidal phase and thus be less available for the enzymatic reaction. Maximum activity of the lipoxidase preparation would, therefore, only be apparent when the major portion of the sodium linoleate is in the soluble phase. If one compares the relative activity reported by Holman for crystalline lipoxidase with the light transmission curve for sodium linoleate at varying pH values, a re-

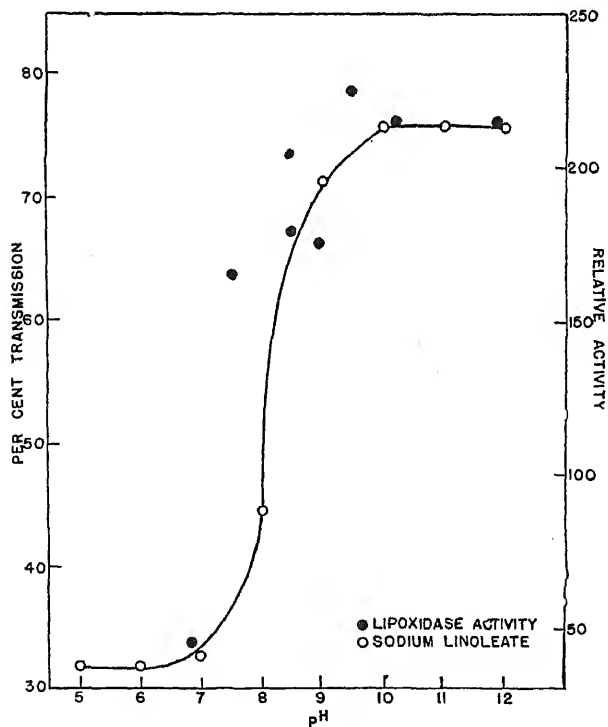


Fig. 7. The absorption of light by a sodium linoleate solution at varying pH values as compared with the relative activity of crystalline lipoxidase reported by Holman (2).

markable similarity exists between the 2 curves. It is the author's opinion that the optimum pH value of 9.4 reported by Holman does not indicate the true optimum for activity of soy bean lipoxidase, but merely indicates the pH value at which the maximum concentration of sodium linoleate exists in the soluble phases and is thus available to the enzyme. This objection has been eliminated in the present experiments since methyl linoleate has been used, and this does not pass through a series of colloidal-soluble phases depending on the change in the pH value of the solution.

ACKNOWLEDGMENT

The author wishes to express his appreciation to Professor James B. Sumner for his advice in this investigation and to the Rockefeller Foundation for financial assistance.

SUMMARY

Spectral changes in methyl linoleate during oxidation with soy bean lipoxidase indicate that pH 6.5 and 25°C. are optimal for maximum enzymatic activity. The activity is fairly constant between pH values of 6 and 7 at 20°C. At 30° and 40°C. the enzyme is more stable at pH 7 than in acid or alkaline solutions. Solutions of pH 8 are more destructive than solutions of pH 6.

Increasing the temperature above 30°C., or the pH value above 7, results in a gradual loss of lipoxidase activity, the effect being most noticeable where a high temperature is combined with a high pH value.

Data have also been presented to indicate that the optimum pH of 9.4 reported by Holman (2) for crystalline lipoxidase is probably not the true optimum for enzymatic activity but merely the pH value where most of his substrate, sodium linoleate, existed in a soluble phase and was thus available to the enzyme. Holman has suggested this effect of high pH on his substrate but has made no distinction between the effect the change in pH would have on his substrate, with its corresponding effect on the activity of the enzyme and the effect of the pH on the enzyme preparation itself.

The optimum conditions for lipoxidase activity are, therefore, probably pH 6.5 and a temperature of 25°C.

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The Extrahepatic Conversion of Carotene to Vitamin A¹

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INTRODUCTION

The conversion of carotene to vitamin A is a function which has been assigned to the liver. The evidence in support of this theory has been reviewed by Mattson *et al.* (1) and labeled as, "far from satisfactory." These workers conclude that the site of conversion of carotene to vitamin A in the rat is the intestinal wall. The evidence presented in support of this view is based on results obtained by the use of vitamin A-deficient rats and may be questioned on the grounds that the site of this conversion has been altered by the deficiency state of the animal. In view of this possibility, it was believed worthwhile to study this *in vivo* conversion process in rats which had not been depleted of vitamin A, by determining whether the liver were necessary for the conversion of carotene to vitamin A.

METHODS

In general, the method used for this investigation was to feed carotene to rats, without hepatic circulation, hereafter referred to as "ligated" rats, and to note changes in the serum vitamin A levels. The rats used were descendants from the Wistar strain and weighed between 150 and 200 g. All rats were fasted 24 hrs. prior to administering carotene. The technique used to obtain "ligated" rats was a slightly modified procedure of that described by Whitaker (2) for portal vein ligation. A ligature was applied to partially ligate the portal vein. This was necessary to stimulate the development of collateral circulation. At the same operation, a second ligature was knotted loosely around the portal vein, hepatic artery and common bile duct and the ends carried through the lateral abdominal wall and secured to the skin. Ten days later the ligature ends were freed from the skin and pulled in such a manner that the knot was tightly closed about these vessels. This operation constituted the second stage ligation. The common bile duct was included in the second ligature in order to insure as complete a

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TABLE I
*Vitamin A Serum Levels of Normal and "Ligated" Rats after Oral
 Administration of 20,000 I.U. of Carotene*

Rat no.	Basal	γ -% vitamin A in serum	
		Hours after carotene administration	
		6	8
"Ligated" rats			
1	7	30	
2	8	24	
3	8	24	
4	13	25	
5	10	25	
6	21	34	
7	20	34	
8	20	49	
9	10		20
10	14		30
11	9		32
Normal rats			
1-N	20	34	
2-N	15	30	

TABLE II
*Vitamin A Serum Levels of "Ligated" Rats after Oral Administration
 of 1 g. of "Tween" 40 in 4 ml. Water*

Rat no.	Basal	γ -% vitamin A in serum	
		Hours after administration of "Tween"	
		6	8
1-C	16	15	
2-C	13	15	
3-C	9		11
4-C	8		11

ligation as possible of all afferent blood vessels of the liver. Immediately after the second stage ligation a basal blood sample was taken by cardiac puncture. This was followed by the oral administration of 20,000 I.U. of carotene in oil² which was mixed in 4 ml. of water with the aid of "Tween" 40.³ Similar quantities of carotene were fed to unoperated, normal rats. In addition, a mixture of "Tween" 40 in water was given to "ligated" rats for control purposes. Blood samples were taken by cardiac puncture at the end of either 6 or 8 hrs. after feeding the carotene. Vitamin A and carotene were determined in the blood serum by the method of Bessey *et al.* (3).

The adequacy of the ligation of the afferent blood vessels of the liver was checked on all rats by an intracardial injection of India ink (1:1 dilution) after taking the final blood sample. The livers were removed from rats No. 1, 4, 8, and 9, 2-3 mins. after the injection, and histological sections made to determine whether ink could be detected in them.

RESULTS

From the gross examination of all livers of the "ligated" animals after the injection of India ink, it was apparent that little or no blood was flowing through the liver. The histological examination of the liver sections showed a few small areas of ink which probably came *via* small blood vessels in adhesions, since ink was found in only small amounts about the periphery of the livers. In comparing the India ink content of livers of normal rats with that of "ligated" ones, one is justified in estimating that the livers from the "ligated" group contained only 10% as much ink as those from normal animals. This, therefore, indicated that only a small amount of blood was flowing into the livers of these animals during the experimental period.

It may be noted from Table I that there was an increase in the serum vitamin A levels of the "ligated" and normal rats either 6 or 8 hrs. after the administration of carotene. The control rats showed no increase in serum vitamin A after the administration of a mixture of "Tween" 40 in water. (See Table II). It is interesting to note that no carotene was detected in the serum of the normal or "ligated" rats after carotene feeding.

DISCUSSION

To adequately prove that the liver is not necessary for the conversion of carotene to vitamin A it must be shown that the carotene administered did not come in contact with liver tissue. This has not been demonstrated to perfection in this study since small amounts of blood and therefore carotene did gain access to the liver, but this amount is so small that it seems unlikely to be of sufficient magnitude to explain the observed increases in vitamin A levels. Secondly, it must be demonstrated that the increase in vitamin A levels is due strictly to the carotene administered. That the rise in vitamin A was due to the carotene, and not to extraneous factors, can be seen from the failure of the vita-

² The carotene used in this study was contributed by Wyeth, Inc.

³ The "Tween" 40 was contributed by the Atlas Powder Company.

min A blood levels to increase in the control group. It seems logical, therefore, to conclude that the conversion of carotene to vitamin A can take place in the rat without hepatic circulation and that the liver is not essential for this transformation.

The coincidental finding that the increase in vitamin A level of the blood of normal and "ligated" rats is about the same after feeding 20,000 I.U. of carotene should not be interpreted as signifying that the rate of conversion in the two groups of animals is the same. Such factors as emptying time of stomach, absorption, and liver storage make it impossible to quantitatively compare the two groups of animals.

The above results lend confirmation to the thesis of Sexton *et al.* (4), Mattson *et al.* (1), and Wiese *et al.* (5), that the conversion of carotene to vitamin A can take place in sites other than the liver.

The failure to find detectable amounts of carotene in the blood of normal or "ligated" rats after the oral administration of carotene may explain why Sexton *et al.* (4) and Mattson *et al.* (1) were unable to find carotene in the liver of rats. The presence of this compound in the intestinal wall after similar feeding experiments (1) indicates that it may be retained there for the conversion process.

In conclusion, it must be stated that evidence presented in this paper does not rule out the liver as an auxiliary site in the carotene to vitamin A conversion process, but it does show that the conversion can take place outside this organ.

SUMMARY

1. Rats without hepatic circulation show an increase in their serum vitamin A levels 6 or 8 hrs. after oral administration of carotene.

2. Evidence is presented to show that the carotene to vitamin A conversion can be an extrahepatic function in the rat.

3. No carotene is detectable in the blood serum of normal rats or rats without hepatic circulation 6 or 8 hrs. after oral administration of 20,000 I.U. of carotene.

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An Unknown Effect of Amino Acids

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In the course of experiments necessitating the precipitation of zinc in a solution of L-alanine, in which the metal could be easily determined after ashing, the following observation was made: Small quantities of zinc, which can be precipitated without difficulty at the same concentration from aqueous solution, are not precipitated by Na_2S in the presence of alanine, either in slightly acid, neutral or alkaline solution. Even freshly prepared ZnS dissolves in alanine solution.

Further examination showed that the effect of the α -aminopropionic acid can be observed also with all other α -amino acids investigated: α -aminoisobutyric acid, L-arginine, L-aspartic acid, L-histidine, L-glutamic acid, glycine, L-lysine, DL-phenylalanine, DL-serine, L-tryptophan, L-tyrosine. The same is true for mixtures of amino acids as they are present in an acid hydrolyzate of casein, or a tryptic digest of fibrin, still containing peptides. It also applies to silk peptone and the mixture of albumoses known as Witte peptone.

Furthermore, it turned out that the phenomenon of the solubility of ZnS in solutions of amino acids and peptides can be extended to other metal sulfides, *e.g.*, MnS , CoS , NiS , FeS , and CuS . There is no difference between the behavior of racemic and optically active α -amino acids, either naturally occurring or synthetically available. However, as will be pointed out in the experimental part, differences exist in some cases.

The effect which, as far as we know, has not been described previously, is fairly independent of pH. Simple α -amino acids and peptides appear to mask the reactions of certain metals in such a way that their normally insoluble sulfides are solubilized. The metals in question include those present in metal proteins and functioning as essential activators of various enzyme systems, *i.e.*, those generally characterized as bioelements. The extensive role played by these metals in

various biological processes can be appreciated from their discussion by Abderhalden (1) and Scharrer (2).

Supplementary experiments were devised to show the effect of the two proteins casein and ovalbumin (in the form of their Na salts) toward metal sulfides. They were found to behave in an analogous manner to the simple α -amino acids and their solubilizing action was particularly marked in the case of Co, Cu, and Fe. Nevertheless, it is not advisable to attach too much value to the results obtained with proteins, since, in contrast to the monomolecular amino acids, they are colloidal and may prevent some precipitation reactions by virtue of their known action as protective colloids. Thus, their solubilizing effect for ferric hydroxide, as well as cuprous and cupric hydroxides, has been known for a long time. Whether formation of undissociated salts according to Rona and Michaelis (3), or specific reactions, such as the formation of coordination compounds and strong polarizing power in the sense of Bersin (4), are involved, must be left undecided (5). Nowadays, no sharp line of demarcation is any longer drawn between crystalloids and colloids; only progressive increase in particle size is taken into account. However, colloidal metal sulfides are possibly formed and are not precipitated by the protein colloids under the experimental conditions used. For the time being it is preferable to consider the typical effect of simple α -amino acids and peptides apart from that of the proteins. Reaction of the simple amino acids (*Zwitterions*) as acids can be ruled out because the pH of the mixture is alkaline ($\text{pH} = > 8$). The basic character cannot be a defining factor either.

The principle applies equally to other α -amino acids, such as DL- α -aminophenylacetic acid and D-glucosaminic acid, both of which have not been observed in nature. The same is true for α -aminoisobutyric acid mentioned in the experimental part.

Separate descriptions of experiments made with racemic and optically active forms are superfluous, as the effects are exactly the same.

Results obtained with a β -amino acid were analogous to those of the α -acids. β -Alanine was used because of its importance as a product of hydrolysis of the naturally occurring carnosine, anserine and pantothenic acid. Thus, the phenomenon described is not limited to α -amino acids. A certain solvent action could also be detected in the case of free D-glucosamine, although it was less pronounced than in the other examples cited. As glucosamine belongs to the sugar group, the possible influence of the increased number of hydroxyl groups must be considered.

The more or less well-defined complex nature of the metal salts of α -amino acids, for instance, of Ni and Cu salts, does not suffice to explain the phenomenon. These metal salts, which are considered to

have a chelate type of structure, are decomposed by H_2S , a reaction utilized in the isolation of amino acids. Furthermore, their complex character is so slight that, *e.g.*, the Cu salts of the amino acids are decomposed even by NaOH (6). Fe complexes, as well as those of Mn and Cu, of peptones and albumoses have been known for decades (ferric albuminate and peptonate, Mn albuminate, and the Cu complexes responsible for the biuret reaction). However, all of these are unstable to ammonium sulfide (7), which may be one of the reasons for overlooking the phenomena described in this paper. The value of the solubility product in itself is not relevant. "Solutions" of, *e.g.*, ZnS , NiS , and CoS in an amino acid form much more readily than those of FeS , although the solubility product of the latter is 3.7×10^{-19} , while that of the other three sulfides is 1.2×10^{-23} , 1.4×10^{-24} , and 3×10^{-26} , respectively, all at 18°C . (The experiments recorded here were made at room temperature, between 18° and 23°C .) It appears that the solutions of the sulfides in α -amino acids are true solutions, as far as this can be deduced from their stability, in some cases for several days, their absolute clarity and the lack of a Tyndall effect at ordinary observation. The presence of a colloidal solution in the usual sense seems unlikely, as no flocculation takes place with reagents which promptly precipitate metal sulfides in supersaturated solutions. Another point in favor of a true solution process is the fact that freshly prepared solid sulfides dissolve in solutions of amino acids. As an example, NiS and MnS , as well as ZnS , dissolve in $1.5\ M$ alanine solutions.

The use of Na_2S is not essential. The same effect is produced with ammonium sulfide or soluble alkali and alkaline earth polysulfides. However, for the reasons stated on p. 154, differences from the control experiments run without amino acids are not always detectable. More or less well defined solubilizing effects can furthermore be observed for sulfides of other metals such as Cd, Pb, Tl, and Sn, although the phenomena relating to these elements require further investigation.

At the present time, nothing definite can be said about the mechanism of the reaction. In particular, a coordination of metal sulfide and amino acid, or the position of S in amino acid complexes, cannot be specified. The assumption that Na_2S acts according to the equations $\text{Na}_2\text{S} + \text{H}_2\text{O} = \text{NaHS} + \text{NaOH}$ or $\text{NaHS} + \text{H}_2\text{O} = \text{H}_2\text{S} + \text{NaOH}$, so that the respective metallic hydroxides formed with simultaneous liberation of H_2S are dissolved by the amino acids, can be rejected, among other reasons, on the following grounds: Cu salts of the amino acids

are decomposed by excess NaOH to yield a precipitate of blue $\text{Cu}(\text{OH})_2$ (6). Cu, Ni, and Co salts of amino acids give a sulfide precipitate on treatment with H_2S . Excess alkali is not required for the occurrence of the phenomenon described. The effect is equally noticeable when equivalent amounts of Na_2S and metal salt react in the amino acid solution at a pH of approximately 7. NiS and CuS, freshly precipitated from the Ni salts and Cu salts of leucine, gradually dissolve in alanine or glycine solution. They dissolve less readily when NaOH is added. The hydroxides of Fe^{++} , Mn^{++} , Cu^{++} , Co^{++} , and Ni^{++} are not precipitated in 1.5 *M* alanine or glycine solutions, but addition of Na_2S leads to the formation of "sulfide complexes." This can be shown by typical color changes, characteristic for the respective metal (*cf.* Table III). Except in the case of Cu, all the solutions remain clear and transparent for 24 hrs. Almost throughout the series those of Ni are colorless, those of Cu golden yellow, those of Co colorless or pale brown, completely different from the shades of the metal compounds of the amino acids in question. In the case of Fe, the color varies from emerald green to olive brown, depending on the amino acid used (*cf.* Table III). Another point disproving the presence of sulfur-free metal salts in the amino acid solutions, is the limited stability observed in some experiments, in which the metal sulfide was precipitated on long standing. This would be in accord with the assumption that sulfido complexes may exist, comparable to the known aquo complexes, with the S atom either inside¹ or outside the complex nucleus. It is known from the chemistry of the cobaltammines that SH-ions attack the metal forming the central atom of a closely bound complex and thus cause the gradual collapse of the entire framework. A pronounced complex-forming ability is common to all bioelements.

Fresenius (8), in his classical studies, made manifest the smallest amount of the respective metals which can be precipitated as their typical sulfides. The figures cited in the experimental part show that 40–400 times this quantity is still held in solution by the amino acid. The presence of the amino acids or peptides in considerable excess and rather concentrated solution appears to be a decisive factor. Such

¹ *Cf.* A. F. Wells, *Structural Inorganic Chemistry*, p. 521, Oxford Univ. Press, London, 1945.—The thioether compounds of Pt of formula $(\text{R}_2\text{S})_2\text{PtX}_2$ (see *e.g.* J. W. Mellor, *Comprehensive Treatise on Inorganic and Theoretical Chemistry*, XVI, p. 275, Longmans, Green, London, New York, Toronto, 1937) belong to a totally different type.

conditions prevail in living cells. Since the metals encounter H_2S during metabolic processes, the possibility must be taken into account that amino acids and peptides, which are always present in comparatively large excess, prevent the precipitation of essential biometals as insoluble sulfides. The precipitation of sulfides of other metals, not considered as bioelements, may be prevented equally as well. This fact may be of importance for nutritional and pharmacological problems after accidental or intentional ingestion. The insight gained into the effect of amino acids also reflects on the general problem of the status and motion of elements in living organisms. Further interest attaches to the phenomenon in its relation to the chemistry of the soil, plankton, saproplankton, well and river water, and stagnant waters. Sulfide is formed by the decomposition of organic matter and reduction of sulfates, especially CaSO_4 . The sulfide reacts with iron and thus affects the cycle of this element. This was pointed out as early as 70 years ago by Hoppe-Seyler (9), who also mentioned the possible role of Mn, stressed later by Bertrand (10). Further knowledge of the Mn metabolism of bacilli, plants and humus has accrued since then (11). The specific importance of Zn for the growth of molds in synthetic nutrient media was discovered by Raulin in 1869 (12).

The similar effect of amino acids toward metallic salts of organic derivatives of H_2S deserves consideration. It can be observed that insoluble xanthogenates of type formula $(\text{C}_2\text{H}_5\text{O} \cdot \text{CS} \cdot \text{S})_2\text{M}$ and, in particular, insoluble mercaptides of various bioelements have been found to be soluble in amino acid solutions. Mercaptans, such as methyl, ethyl and butyl hydrosulfides, are products of metabolism. Neuberg and Nord (13) have shown that mercaptans form whenever aldehydes come in contact with H_2S in glycolytic systems, whereby the easily decomposed thioaldehydes are phytochemically reduced to the more stable thioalcohols.

EXPERIMENTAL

Of the 6 metals investigated, ZnS and CuS can be precipitated in the wet way by H_2S ; NiS, CoS, MnS, and FeS are prepared with ammonium sulfide. To work under comparable conditions, Na_2S was used in all experiments. This reagent has several advantages. As it is always applied in excess, an alkaline pH is invariably obtained, even in the presence of amino acids. More important, however, Na_2S is available in pure form and of constant composition. The presence of true monosulfide, which may usually be neglected in ordinary inorganic analysis, is of importance for the processes under consideration; these, in spite of their apparent simplicity, have a

somewhat subtle character. It is difficult to comply with this condition in the case of ammonium sulfide, since polysulfides may dissolve CuS , NiS , and CoS to yield salts of thioacids (14) such as $\text{Na}_2\text{Cu}_2\text{S}_7$, which has been isolated. Furthermore, any ammonium thiosulfate, formed in ammonium sulfide, may garble the results.

Commercially available $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ can be used, provided it is fresh and gives a perfectly colorless solution in water. If this is not the case, it must be prepared from NaOH with exclusion of air. $\text{Ba}(\text{SH})_2$ made from pure $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$ is also applicable if sulfate ions are absent. Under no circumstances should S be precipitated on addition of dilute HCl to the sulfide reagents. In all control experiments where the solution of the amino acid has been replaced by the same quantity of distilled water, the Na_2S must precipitate the metal sulfide from the metal salt solutions in such a way that the supernatant liquid no longer contains metal ions. Complete precipitation is effected by adding a few drops of a mixture of NH_4OH and NH_4Cl or $(\text{NH}_4)_2\text{SO}_4$ in equimolecular amounts (designated as "Ammono" solution in the tabulated results). A few drops of a mixture of ethylamine, derived from alanine, and ethylamine hydrochloride in the ratio of 1 g.:2 g. in 25 cc. water promotes flocculation of metal sulfides in a manner exactly analogous to the "Ammono" solution in the case of Zn , Co , Ni , and Cu . In the case of Fe the action is slightly weaker than, but analogous to, the other effects after warming to 50°C . Both reagents do not prevent the solution of freshly precipitated metal sulfides in the presence of alanine and other amino acids. The amino acids must be pure; in particular, they must be free from iron which is frequently present in commercial preparations. A green or olive brown shade of the iron complexes (see Tables II and III) otherwise lead to atypically colored solutions of the other bioelements.

The nature of the anion bound to the metal is irrelevant both for the precipitation by Na_2S and the non-precipitation in the presence of amino acids. No distinct difference between chlorides, sulfates, nitrates, acetates, *etc.*, can be observed. The ZnSO_4 solution can be replaced by $\text{Zn}(\text{NO}_3)_2$, ZnCl_2 , Zn acetate, Zn dl-lactate , Zn D-ribonate , $\text{Zn } \alpha\text{-glycerophosphate}$ and $\text{Zn D-fructose-6-phosphate}$ without altering the phenomenon. At the most, slight differences may occur in the time required to flocculate or dissolve the ZnS . Table I shows the results obtained with ZnSO_4 and *DL*-alanine.

TABLE I

$M/100 \text{ ZnSO}_4$	1.5 M Alanine	$M/10 \text{ Na}_2\text{S}$	
cc.	cc.	cc.	
1	2	0.5	Turbid at first, clear solution after 3 mins., stable 40 hrs.
2	2	0.5	Becomes clear after 5 mins.
2	1	0.5	Perfectly clear after few mins.
3	2	0.6	Slowly becomes clear.

In all cases control experiments in which the alanine solution was replaced by the same volume of distilled water gave flocculent precipitates of ZnS , immediately or on shaking.

The freshly precipitated ZnS can be redissolved completely within a few mins. on adding 2 cc. of 1.5 *M* alanine solution. Without addition of the amino acid neither dispersion nor peptization of the ZnS can be observed.

The solutions obtained in the presence of alanine remain perfectly clear for about 40 hrs.; then some turbidity appears, but no precipitation or separation of ZnS occurs.

TABLE II

		1.5 <i>M</i> Alanine	<i>M</i> /10 Na ₂ S	
cc.		cc.	cc.	
(a) <i>M</i> /100				
Zn acetate	1	2	0.5	Clear after 3 mins. Remains clear 48 hrs.; also with "Ammono" soln. Control with 2 cc. water flocculates on shaking.
	2	2	0.5	Clear after 5 mins. Remains clear 40 hrs.; also with "Ammono" soln. Slightly turbid after 48 hrs.
	2	1	0.5	Clear after 5 mins.
	3	2	0.6	Clear after 10 mins.
(b) <i>M</i> /100				
Mn acetate	0.2	4	0.5	Remains clear for hours; also with "Ammono." Control with 4 cc. water pptd. with "Ammono."
	0.4	4	0.5	Exactly as above.
	0.6	4	0.5	Exactly as above.
	1	4	0.5	Remains clear for 3 mins. after adding "Ammono," then becomes turbid.
(c) <i>M</i> /1000				
Co nitrate	1	2	0.5	Darkens, but remains clear for hours. After 18 hrs. soln. is turbid, but no ppt. has formed. Control with 2 cc. water soon precipitates CoS.
	2	2	0.5	Exactly as above.
	4	2	0.5	Exactly as above. Dilution with the same volume of water shows that no ppt. has formed after 18 hrs.

TABLE II—*Continued*

		1.5 M Alanine	M/10 Na ₂ S	
	cc.	cc.	cc.	
(d) M/1000				
Ni acetate	2	2	0.5	Gradually darkens, but no ppt. is formed in 40 hrs., even with "Ammono." In the control NiS rapidly precipitates without "Ammono."
	4	1	0.5	Exactly as above.
(e) M/100				
Cu sulfate	2	1	1	Yellowish brown solution, becomes turbid after $\frac{3}{4}$ hr. Control flocculates on shaking.
	4	1	1.5	Dark but clear solution. Becomes turbid after $\frac{3}{4}$ hrs.
(f) M/1000				
FeCl ₂ or FeCl ₃	1	2	0.3	Olive colored clear solution for hours, also with "Ammono." Control with water instead of 1.5 M alanine ppts. on adding "Ammono." Dilution with water enhances the difference.
	2	2	0.5	Exactly as above.

Remarks: (a) Freshly precipitated ZnS dissolves completely on adding the alanine solution and remains clear, also with "Ammono" solution. *E. g.*, the ppt. of 2 cc. Zn acetate + 0.5 cc. Na₂S as above is dissolved by 2 cc. 1.5 M alanine soln. in 14 mins. and is still perfectly clear after 40 hrs. Similarly, ZnS obtained from 2 cc. ZnSO₄ as above, centrifuged off and washed with water, dissolves completely with 2 cc. of amino acid solution. After two washings on the centrifuge the precipitate no longer dissolves completely unless NaOH or Na₂S is added. Similar results were obtained with other Zn salts.

(b) Freshly prepared MnS dissolves on adding 4 cc. 1.5 M alanine. After 5 mins. the solution becomes slightly turbid but is not precipitated for hours.

(c) The same results were obtained with a solution of 5 cc. M/200 Na cobaltinitrite and 1 cc 1.5 M alanine, or 2 cc. 2 M glycine + 0.5 cc. M/10 Na₂S with or without "Ammono" solution. The dark but transparent solution shows a slight precipitate after 18 hrs.; while the control with 1 cc. water instead of the amino acid soon yields a black precipitate. Doubling the amount of 1.5 M amino acid, the solution is still clear after 24 hrs.

(e) Addition of 2 cc. 1.5 *M* alanine to the precipitate obtained with 4 cc. CuSO_4 , 1 cc. water, and 1.5 cc. Na_2S yields an opalescent, but not perfectly clear, greenish yellow solution. The same results as those tabulated are obtained with less concentrated reagents and with "Ammono."

(f) In this case it is irrelevant whether Fe_2S_3 or a mixture of $2\text{FeS} + \text{S}$ is formed. The solutions containing amino acids are usually olive brown or else emerald green. The control solutions in which an equal amount of distilled water replaces the alanine solution are invariably dark green up to the moment of precipitation of the flocculent black FeS .

The same type of experiment was extended to other metals and other amino acids.

Table II shows the wide limits within which the effect may be observed and, to a certain extent, its independence of the valency (Co, Fe) and the anion, even in the case of complexes such as sodium cobaltinitrite.² Table III brings out some differences observable by varying the amino acids or substituting peptones and proteins for them. It may be worth mentioning that Fe, Co and Mn give differently colored solutions with different amino acids, a fact which may be due to formation of different complexes. Physiologically, the variations in stability in the case of Cu and Fe appear of interest. The similar effect of amino acids toward metallic salts of benzylmercaptan as an organic derivative of H_2S is outlined in Table IV. Benzyl mercaptan was chosen because it is more agreeable to handle than aliphatic mercaptans.

All experiments were made in test tubes; if the samples are to be kept for some time they must be closed tightly with rubber stoppers to avoid contamination by CO_2 with formation of free H_2S , atmospheric oxidation of the latter and consequent separation of S. It is advisable to first boil the stoppers in water and thereby eliminate as far as possible S derived from the vulcanization. This procedure reduces the formation of polysulfides which might obscure the phenomenon (see p. 154). To make the results more convincing the amount of Na_2S applied was persistently in excess of that required for complete precipitation of the metal. A possible effect of NaOH which might be imagined as derived from Na_2S (see p. 151) is irrelevant, since ZnS is precipitable from Na zincate by both H_2S and Na_2S .

No account need be taken of slight precipitates which may appear when the first drops of the amino acid solutions or the solutions of

² The precipitation of Co from some other cobalt complexes of the purpureo or roseo series by ammonium sulfide is known and reported to yield Co_2S_3 (15),

TABLE
The Effect of Various

In all cases 0.5 cc. $M/10$ Na_2S was added to a solution of 1 cc. of the metal salt +
were precipitated

	$M/100$ Zn acetate	$M/1000$ Co nitrate	$M/1000$ Ni acetate
DL-Alanine 1.5 M	Clear after few mins.	Dark, but clear for hrs.	Gradually darkens, but no ppt. forms for hrs. even with "Ammono" soln.
DL- α -Aminoisobutyric acid (2.06 g. in 20 cc. dist. H_2O)	Clear, stable 1.5 hrs., also with "Ammono" soln.	Dark, but transparent, stable 1.5 hrs., also with "Ammono" soln.	Clear, stable 1.5 hrs., also with "Ammono" soln.
L-Arginine HCl (4.2 g. in 10 cc. 2 N NaOH)	Colorless and clear for hrs., also with "Ammono."	Darkens somewhat, but remains clear, also with "Ammono."	Colorless and clear for hrs., also with "Ammono."
L-Aspartic acid (2.66 g. in 10 cc. 2 N NaOH)	Clear at first, but soon becomes turbid.	Colorless, perfectly stable for hrs.; pale pink, but clear and stable for hrs. with 2 cc. Co soln.	Clear, colorless soln., stable, for hrs.
L-Cystine (2.4 g. in 10 cc. 2 N NaOH)	Perfectly colorless and clear for hrs.	Perfectly clear, light green, stable for many hrs.	Perfectly colorless and clear for hrs.
L-Glutamic acid (2.95 g. in 10 cc. 2 N NaOH)	Clear at first, later turbid.	Clear for hrs., gradually darkens.	Clear and almost colorless for hrs.
Glycine 1.5 M	Clear after few mins.	Dark, but no ppt.	Clear and perfectly colorless soln., also with "Ammono."
L-Histidine HCl $\cdot H_2O$ (4.2 g. in 30 cc. H_2O + 10 cc. 2 N NaOH)	Absolutely clear and colorless, also with "Ammono." Stable and transparent for hrs.	Absolutely clear and colorless, also with "Ammono." Stable and transparent for hrs.	Absolutely clear and colorless, also with "Ammono." Stable and transparent for hrs.
L-Lysine HCl $\cdot H_2O$ (4 g. in 10 cc. 2 N NaOH)	Perfectly clear for hrs.	Clear and colorless for hrs., also with "Ammono."	Colorless and absolutely clear for hrs.
DL-Phenylalanine (3.3 g. in 20 cc. 2 N NaOH)	Flocculent ppt.	Clear, pale brown, also with "Ammono."	Perfectly clear and colorless for hrs.
DL-Serine (2.1 g. in 10 cc. 2 N NaOH)	Clear and stable for hrs., also with "Ammono."	Clear and stable for hrs., also with "Ammono."	Clear and stable for hrs., also with "Ammono."
DL-Tryptophan (4.08 g. in 10 cc. 2 N NaOH)	Clear, becomes turbid after 1.5 hrs.	Clear, almost colorless, stable for hrs., also with "Ammono."	Clear almost colorless, also with "Ammono," stable for hrs.
L-Tyrosine (3.62 g. in 20 cc. 2 N NaOH)	Clear at first, then turbid; remains perfectly clear with thrice the amount of tyrosine.	Darkens slowly, but remains clear for hrs.	Clear and colorless for hrs.
β -Alanine (2 g. in 12 cc. water)	Turbid at first, becomes perfectly clear and stable for hrs., also with "Ammono."	Dark, but clear for hrs., also with "Ammono."	Dark, but clear for hrs., also with "Ammono."
Silk peptone (15% soln.)	Fairly clear.	Darkens, but remains perfectly transparent.	Darkens but remains perfectly transparent.
Witte-Peptide (15% soln.)	Turbid at first, becomes clear on adding more peptone	Darkens, but remains clear.	Darkens somewhat, but remains clear.
Casein (Hammarsten) (2.5 g. + 0.5 g. $NaHCO_3$ in 50 cc. water)	Slightly turbid.	Clear, stable 24 hrs.	Clear, slightly turbid after 24 hrs.
Ovalbumin (6 g. + 1 g. $NaHCO_3$ in 100 cc. water, shaken in the cold, then centrifuged)	Clear, also with "Ammono."	Clear, also with "Ammono."	Clear, also with "Ammono."
Acid casein hydrolyzate (dry powder-pH 6.5) (15% H_2O soln.)	Turbid.	Darkens somewhat, but remains clear for hrs.	Perfectly clear, almost colorless for hrs.
Tryptic fibrin digest (dry powder-pH 6.9) (15% H_2O soln.)	Slightly turbid at first, becomes perfectly clear on standing.	Darkens, but remains clear.	Perfectly clear and transparent for hrs.

* Zn acetate ppts. proteins; therefore a turbidity appears at first. This may be centrifuged off,

III

Amino Acids, Peptides, Etc.

2 cc. amino acid solution. Controls made with water instead of the amino acid solution as expected.

<i>M</i> /100 Mn acetate	<i>M</i> /1000 Fe Cl ₂	<i>M</i> /100 CuSO ₄	Remarks
Clear soln.	Clear for hrs., also with "Ammono" soln.	Yellowish-brown soln., becomes turbid after 45 mins.	
Turbid	Transparent soln. at first, also with "Ammono" soln., but soon flocculates.	Clear yellow soln., flocculates slightly after 1.5 hrs.	Due to the slight solubility of this amino acid, this soln. is more dilute; therefore 3 cc. are used in this series.
Becomes turbid.	Flocculent ppt.	Remains clear, pale yellow for 20 mins., also with "Ammono," then turbid.	
Clear, colorless soln., stable for hrs.	Rapid pptn.	Colloidal soln.	
Flocculent ppt. of cystine caused by the NaOH; partially dissolves on adding larger excess of sulfide.	Rapid pptn.	Deep blue soln. before adding Na ₂ S, becomes yellowish with Na ₂ S, slightly turbid, but no ppt., even with "Ammono."	
Clear and almost colorless.	Ppts. but slowly, even with "Ammono" not at once.	Becomes turbid rapidly. Colloidal soln.	
Clear at first, but soon becomes turbid and opalescent.	Dark but clear soln., also with "Ammono"; olive color.	Yellowish brown soln., clear at first then turbid; green opalescence.	
Absolutely clear and colorless, also with "Ammono," stable and transparent for hrs.	Stable, green soln., clear for hrs., also with "Ammono."	Turbid.	The histidine dissolves on warming. In this series 5 cc. of the lukewarm soln. are used. Controls with 5 cc. water show that the dilution is not relevant.
Perfectly clear, almost colorless at first, then pale pink, also with "Ammono."	Ppts. immediately	Clear yellow at first, but soon becomes turbid.	
Flocculent ppt. soon forms.	Clear green soln. for 10 mins. then flocculent ppt.	Clear at first, then flocculent ppt.	
Clear and stable for hrs. also with "Ammono."	Ppts. rapidly.	Soon becomes turbid.	
Clear, also with "Ammono," stable for hrs.	Flocculent ppt.	Colloidal soln., flocculent after 1.5 hrs.	
Clear and almost colorless for hrs.	Ppt.	Ppt.	
Milky turbidity at first, then flocculent ppt.	Clear, dark green for hrs., also with "Ammono."	Clear yellow for 30 mins., then turbid, no flocculation.	
Darkens but remains perfectly transparent.	Remains clear for hrs., greenish.	Remains clear, yellowish brown.	
Remains clear, and almost colorless	Remains clear, dilution yields emerald green, transparent soln.	Remains clear for 3 mins., then turbid.	
Becomes turbid after 2 hrs.	Remains clear 24 hrs.	Remains clear 24 hrs.	The casein soln. was centrifuged after standing 12 hrs.
Gradually ppts.	Clear soln., stable 24 hrs., green on dilution with 12 cc. water.	Clear yellow soln., stable 24 hrs.	4 cc. of the protein soln. was used in this series.
Turbid.	Flocculent ppt. soon gotten with "Ammono."	Flocculent ppt. soon forms.	To get a clear H ₂ O soln., some tyrosine is centrifuged off.
Clear at first, but becomes turbid after 1.5 hrs.	Flocculent ppt. with "Ammono" soln.	Transparent and stable, also with "Ammono" soln.	Tyrosine is centrifuged off to get a clear soln. which gives a distinct biuret reaction.

leaving sufficient Zn in soln. This soln. on adding Na₂S remains clear and transparent for hrs.

their sodium salts are added to the dilute metal salt solutions. Such precipitates readily dissolve as soon as the amino acid is present in excess.

TABLE IV

The Effect of Amino Acids on Metal Mercaptides

In all cases 1 cc. of the metal salt solution, 2 cc. of 1.5 *M* alanine solution, and 2 cc. of the Na mercaptide were used. The same volume (3.5 cc.) of methanol was added to prevent nonspecific turbidities. After some time the amino acid partially crystallizes from the 50% methanol solutions, but the white needles are easily distinguished from the colored precipitates formed when 2 cc. of water replace the alanine solution.

1.25 g. benzyl mercaptan in 10 cc. *N* NaOH +
90 cc. 60% C₂H₅OH

<i>M</i> /100 Zn acetate	Colorless and clear, but control also remains colorless and clear.
<i>M</i> /1000 Co nitrate	Perfectly colorless and stable for days, while the control at once turns red-brown and yields a red flocculent precipitate after 4 hrs.
<i>M</i> /1000 Ni acetate	Perfectly colorless and stable for days, while the control becomes brown at once and yields a flocculent yellow precipitate after 4 hrs.
<i>M</i> /100 Mn acetate	Perfectly colorless and stable for days, while the control yields a yellowish brown flocculent precipitate.
<i>M</i> /1000 FeCl ₂	Very pale green solution, while the control yields a greenish-black flocculent precipitate.
<i>M</i> /100 CuSO ₄	Pale yellow turbidity similar to that obtained in the control experiment.

Note: In all cases the pH is approximately 7.

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SUMMARY

It is demonstrated that the difficultly soluble metal sulfides are held in solution or dissolved by α -amino acids and peptides. This property has been investigated more thoroughly for the 6 bioelements Zn, Ni, Co, Mn, Fe, and Cu. The same effect could be confirmed for a β -amino acid, β -alanine, and for related compounds. There is no difference in the solubilizing ability of optically active and racemic amino acids,

whether they do or do not occur in nature. In principle, the effect is the same for all amino acids investigated; it differs, however, in detail. The solutions are often colorless or show marked deviations in shade from that of the typical sulfide precipitates.

Similar effects have been observed with an organic derivative of H_2S , benzyl mercaptan.

The possible mechanism of the reaction is considered and the biochemical importance of the phenomenon is discussed.

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Dismutation in the Heterocyclic Series. Dismutation of Furfural by Yeast and Related Problems

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INTRODUCTION

Furfural has played a part in the investigations on the influence of yeast upon added substances. No statement can be found in the literature as to whether dismutation into α -furoic acid and furfuryl alcohol is possible, this being the simplest biochemical transformation according to the overall reaction.

Fifty years ago W. Windisch (1) reported that furfural disappears after having been added to a sugar solution fermenting with brewers' yeast. The nature of the transformation was the subject of a later study by Lintner and v. Liebig (2); they found that with brewers' yeast 2 alcohols are formed, furfuryl alcohol and a compound assumed to be furyl trimethylene glycol. Based on the investigations of Neuberg, Hirsch *et al.* (3) Liang (4) elucidated the constitution of the latter compound as being the analogous α -methyl- β -furylethylene glycol. Furfural is one of the most efficient activators of cell-free fermentation (5) as well as hydrogen acceptors. Accordingly, the use of furfural, as recommended by Neuberg and Lustig (6), has been of special value in the preparation of D(-)3-phosphoglyceric acid from D-fructose-2,6-diphosphate. In this oxidation-reduction process the formation of furfuryl alcohol has been proved (6). The fate of furfural in the enzyme system of xanthine oxidase and Schardinger enzyme (7), and of furfuryl alcohol under the influence of various species of acetobacter (8) has not been clarified. Furoin, the condensation product of furfural corresponding to benzoin, yields the glycol hydrofuroin in the same way as its oxidation product furil (9).

In spite of the fact that furfural and furfural derivatives were often used in studies of enzymatic processes, investigation has never been made as to whether this aldehyde or any aldehyde of the heterocyclic series is able to undergo simple dismutation. This can be done by maintaining the experimental conditions described below. They allow

¹ Deceased December, 1947.

the isolation of furfuryl alcohol and α -furancarboxylic acid in the pure state, and also the characterization of both compounds by crystalline derivatives.

Lintner and v. Liebig (2) did not elucidate the character of the oxidation product which corresponds to the hydrogen-rich transformation products mentioned above. Its nature is undefined; it may have been acetaldehyde or acetic acid (10). The authors (2) stated explicitly that furoic acid had not been found.

"Dismutation" and "phytochemical reduction" yield different products. Saturation of double bonds (11), theoretically conceivable in phytochemical reduction, does not take place in simple oxidation-reduction. The presence of tetrahydrofurfuryl alcohol could not be demonstrated.

It was observed as early as 1915 (12) that the potassium ion and the ammonium ion are indispensable for some partial processes of fermentation. Correspondingly, these ions show their influence also in complete glycolytic systems.

Besides the investigations mentioned elsewhere (13), we may refer to the statement of Smythe (14) that NH_4^+ reduces the period of induction in alcoholic fermentation. Neuberg and Lustig (15) have demonstrated that, in general, zymase extracts from top yeast are efficient only in the presence of K^+ or NH_4^+ , and that ineffective extracts can be rendered capable of fermentation by adding boiled yeast extracts if these extracts contain as co-factors, NH_4^+ , Mn^{++} , Mg^{++} , Po_4^{---} , acetaldehyde, and fructose-2,6-diphosphate as well as the co-enzymes. Using similar enzymatic systems, Muntz (16) recently arrived at the conclusion that the above-named ions are responsible especially for the formation of fructose-2,6-diphosphate from fructose-6-phosphate.

Neither K^+ nor NH_4^+ show an influence on the oxidation-reduction process by which furfuryl alcohol and furoic acid are produced. Therefore, it is not necessary to relate experiments by which this dismutation was effected using well-washed bakers' yeast and adding varying quantities of K_2HPO_4 or $(\text{NH}_4)_2\text{HPO}_4$ or the potassium (or ammonium) salt of D-3-phosphoglyceric acid. This negative result is in accordance with the findings of Utter and Werkman (17) that the effect of another mutase, arising with the two phosphoglyceric acids, is also independent of K^+ .

No difference is discernible, either, in the rate of the phytochemical reduction of furfural, whether it is effected with or without addition of K^+ or NH_4^+ . Phytochemical reduction is possible in a cell-free system (18). If it is carried out with non-dialyzed maceration juices from bottom yeast (Piel's brewers' yeast) no promoting effect of the 2 above-named ions can be demonstrated. There exist considerable differences in

the reaction times of "dismutation" and "phytochemical reduction." These can be demonstrated in a simple way in the case of furfural.

Nowadays, phytochemical reduction can be conceived of as a special case of mixed dismutation in the sense of Nord (19). It takes place between the phytochemically reduced substance and the dihydrocozymase. The latter, in turn, obtains its labile hydrogen ("Gärungswasserstoff") from donators formed rapidly and continuously by glycolysis. The role of the original "Gärungswasserstoff" can be specified as the effect of the cozymase-flavine-enzyme combination, probably with the participation of Keilin's cytochromes and the enzymes adapted to the cytochrome cycle (20). The mechanism of the dismutation reaction as an effect of the aldehyde mutase system is discussed by Oppenheimer (21).

EXPERIMENTAL

A. Dismutation

The experiments carried out under varied conditions took essentially the same course. Variation of temperature from 23°C. to 37°C., and variation of furfural concentration from 0.6% to 1.5% were without perceptible influence.

Two hundred eighty g. of Fleischmann's bakers' yeast are suspended in 550 ml. of tap water. Nine g. of pure furfural and 10 g. of CaCO_3 are added. The volume is made up to 1000 ml. The vessel must be stoppered by a fermentation lock, as CO_2 is formed by autofermentation and from the CaCO_3 . After keeping at room temperature for 48 hr., all the furfural has disappeared.

The reaction mixture is refluxed on the water-bath to prevent volatilization of furfuryl alcohol. When lukewarm, it is centrifuged. The residue is washed several times with warm water.

The clear supernatant liquid is saturated with Na_2SO_4 to a concentration of 90%, filtrated if necessary, and extracted with 1000 ml. of ether in several portions, (adding a few drops of ethanol to prevent frothing). This quantity is appropriate because of the considerable hydrotropic solubility of furfuryl alcohol in calcium furoate (22). The light yellow ether extract is dried during 24 hr. over dry Na_2SO_4 and distilled through a fractionating column in such a manner that, by letting it run drop by drop through one of the tubes of a two-neck flask, the furfuryl alcohol is concentrated in a small volume of ether. This ether solution is dried once more over dry Na_2SO_4 . The fractionation yielded, besides a small first running (ethanol), 3.2 g.; b.p. 168–172°C.

Several drops of a high boiling viscous yellow oil remained in the flask. Its dilute alcoholic solution strongly reduced ² Fehling's solution upon heating, and yielded with

² Furfuryl alcohol rapidly reduces Tollens' solution, and, in stronger concentration, also reduces Fehlings' solution slowly.

2,4-dinitrophenyl-hydrazine in HCl solution a semisolid osazone which showed a violet coloration with sodium ethylate, characteristic of the dinitro osazones of dicarbonyl compounds. This may be due to the presence of small quantities of an acyloin formed from materials furnished by autofermented reserve carbohydrates of the yeast.

The main fraction is practically pure furfuryl alcohol. It showed the characteristic behavior towards strong HCl.

The α -naphthylisocyanate compound was prepared for identification. It melted at 132°C. The melting point for the α -naphthylurethane of furfuryl alcohol has been given by Neuberg and Hirschberg (23) as 133°C., by Bickel and French (24) as 129–130°C. Calculated 5.24%; found 5.3% N.

The reaction mixture, freed from furfuryl alcohol, after having been extracted at neutral pH, was rendered acid to Congo by HCl. The extraction with ether was continued until complete. After evaporation of the ether a slightly colored crystal cake remained. It was stirred with a little cold water, filtered by suction and recrystallized from dilute alcohol after addition of charcoal, yielding 3.2 g. of α -furoic acid. Long needles; m. p. and mixed m. p. 132°C.

The characteristic silver furoate may serve for identification purposes. This salt was obtained from the aqueous solution of the ammonium salt with 20% AgNO₃, the yield being practically quantitative. Microcrystalline powder of great stability to light. The salt is obtained in brilliant, long, coarse white needles, after having been dissolved in dilute hot ammonia or pyridine, concentrated, and allowed to cool. C₆H₃O₃Ag; calculated 49.3%, found 49.05% Ag.

The yield of α -furoic acid amounted to 61%, that of furfuryl alcohol to 70%.

The acid can be isolated more readily than the alcohol. In addition to defects inherent in the method, the fact that the phytochemical reduction precedes the dismutation is responsible for the lack of complete correspondence (cf. part B). Since the quantity of fresh yeast used under the chosen experimental conditions is considerable, superimposing of a phytochemical reduction at the expense of autofermentation of carbohydrates of the yeast cannot be avoided entirely.

B. Comparison between Dismutation and Phytochemical Reduction

The following experiments reveal the difference in the rate of the two biochemical processes.

Required reagents: (a) 2% furfural solution, (b) 20% saccharose solution, (c) mixture of 2.5 ml. aniline, 2.5 ml. ethanol, 2.5 ml. glacial acetic acid, and 2.5 ml. water.

SERIES I. TEMPERATURE 25°C.

- (a) 5 ml. furfural solution } + 0.5 g. bakers' yeast.
5 ml. H₂O }
(b) *idem* + 1 g. yeast.
(c) *idem* + 2 g. yeast.
(d) *idem* + 3 g. yeast.
(e) *idem* + 5 g. yeast.

After the times indicated below, 2 ml. are withdrawn and examined by means of the aniline acetate reagent, either directly or after centrifuging. In (e) no more furfural is present after 24 hr., after 36 hr. hardly any furfural remains in (d) either. In all other samples unchanged furfural is demonstrable after 48 hr. and longer periods with diminishing intensity; after 72 hr. (c) still shows traces. In all experiments in which dismutation was incomplete, furfuryl alcohol can also be detected after centrifuging.

SERIES II. SAME AS SERIES I. TEMPERATURE 37°C.

Same results.

SERIES III. TEMPERATURE 25°C.

- (a) 5 ml. furfural solution } + 0.5 g. yeast.
5 ml. saccharose solution }
(b) *Idem* + 1 g. yeast.
(c) *Idem* + 2 g. yeast.
(d) *Idem* + 3 g. yeast.

All experiments show distinct fermentation. After 20 hr. they are practically free from furfural, after 24 hr. completely free. In all samples furfuryl alcohol is demonstrable.

SERIES IV. SAME AS SERIES III. TEMPERATURE 37°C.

Same results.

SERIES V. SAME AS SERIES I, BUT WITH ADDITION OF
0.2 g. CaCO₃ TO EVERY EXPERIMENT

Results as in Ser. I, with the difference that (c) and (d) needed a longer time for completion. All series are set up simultaneously and with the same yeast.

It is evident that furfural is reduced phytochemically much more rapidly and by much less yeast than it is dismuted. Comparison of experiments (c) leads to the estimation of the reaction velocities 3:1.

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SUMMARY

It seems that hitherto it had not been investigated as to whether dismutation by means of yeast can be accomplished with an aldehyde of the heterocyclic series. Furfural, used for the study of many biochemical processes for 50 years, now tested in this respect, undergoes dismutation by fresh bakers' yeast into α -furoic acid and furfuryl alcohol. Neither NH_4^+ nor K^+ have an influence on this reaction. The same holds true for the phytochemical reduction of furfural by fresh top yeast or by maceration juice from bottom yeast. The simplest test demonstrates that phytochemical reduction proceeds by means of less yeast and much more rapidly than does dismutation. The reaction velocities are approximatively 3:1.

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LETTERS TO THE EDITORS

A Note on the Component Enzymes of *Pseudomonas* "Creatinase"

Recently Kopper and Beard (1) reported on the "creatinase" activity of a strain of *Pseudomonas*. On the basis of some of their experiments, they suggested that the breakdown of creatinine by the organism proceeded by way of the hydration of creatinine to creatine and subsequent hydrolysis of creatine. Further evidence in support of this assumption has been obtained.

Two enzymes could be differentiated in heat resistance studies. Exposure of bacterial suspensions to a temperature of 100°C. for 10 min. was required to destroy their ability to convert preformed into total Jaffe-reactive substances, while the further breakdown of the latter was already completely inhibited by incubation at 50°C. for the same length of time.

In cultures of the organism grown in the presence of high concentrations of creatinine and allowed to stand at room temperature for 7-10 days, there appeared regularly a crystalline material, which upon analysis proved to be creatine hydrate. Solutions of 5, 6, 7, and 8%

TABLE I
Effect of High Substrate Concentration on the "Creatinase"
Activity of a Strain of Pseudomonas

Initial amount of creatinine/2 ml.	Amount of Jaffe-reactive substances found after bacterial action		Amount of crystalline creatine hydrate recovered	Creatinine transformed into creatine
	Preformed Jaffe	Total Jaffe		
mg.	mg.	mg.	mg.	per cent
100	30	60	26	51
120	24	60	48	60
140	28	70	90	79
160	24	64	126	85

creatinine, respectively, in distilled water were prepared and dispensed in 2 ml. quantities into test tubes. Suspensions of 6×10^8 organisms were added to each tube. The results are presented in Table I. Control tubes without bacteria showed no change in creatinine concentration during the course of the experiment.

In accordance with the experimental data obtained it seems justifiable to consider *Pseudomonas* "creatinase" as a mixture of two enzymes, a creatinine hydrase and a creatine hydrolase or possibly oxidase. This would suggest that the organism follows in the dissimilation of creatinine a metabolic pathway different from that postulated for *Corynebacterium creatinovorans* by Dubos and Miller (2, 3).

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Application of Paper Chromatography to the Estimation of Free Amino Acids in Tissues

The application of paper chromatography (1) to the analysis of free amino acids in tissues of the rat has been successfully accomplished.

Fresh tissues were minced and homogenized with 5 volumes of hot water in a Waring blender. Sufficient absolute alcohol was added to precipitate all the proteins. The precipitate was centrifuged down, and the clear supernatant was removed and mixed with 3 times its volume of chloroform. Upon centrifugation the clear aqueous layer separated from the alcohol-chloroform mixture. The recovery of amino acids by this procedure was better than 90% as measured by the method of Frame, Russell and Wilhelmi (2).

For chromatography it was necessary to concentrate the extract at least 5-fold (of its volume) after which it was chromatographed on paper, using phenol.

Liver, kidney, spleen, and heart of Wistar rats were analyzed in this manner. The results were remarkably similar for all tissues. Four spots, which corresponded to aspartic acid, glutamic acid, glycine, and alanine, were present in all cases. *The identities of these amino acids were confirmed by acid hydrolysis of these 4 fractions, after elution from the paper, followed by rechromatography with known solutions.*

Further proof on the identity of these 4 spots was obtained by chromatography with collidin.

A quantitative estimation of these amino acids was accomplished by eluting the various fractions from the paper and determining amino acid nitrogen colorimetrically with β -naphthoquinone sulfonate. The 4 amino acids mentioned above accounted for almost all the free amino acid nitrogen present in the extract.

Further work on quantitative analysis of tissues for amino acids is in progress in this laboratory.

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Hyaluronidase Inhibitor in Electrophoretically Separated Fractions of Human Serum¹

It has been shown by Hobby *et al.* (1), McClean (2), Haas (3), and others, that the blood sera of many species contain a non-specific inhibitor of hyaluronidase activity which will act on the enzyme regardless of its source. It would appear that this serum inhibitor is a separate entity from the specific antibody inhibitors that are elicited when hyaluronidase is used as an antigen (4). It has been claimed by Haas (3) that the non-specific inhibitor is an enzyme, a point of view which cannot be considered established (5). McClean (2) stated that there was evidence that the inhibitory action was associated with the pseudo-

¹ This work was supported in part by a grant from the Division of Research Grants and Fellowships, National Institute of Health, U. S. Public Health Service, Bethesda, Maryland.

globulin of the serum proteins; however, the evidence was not given. The inhibitor has not been isolated and identified, and as a step toward this end, human blood serum was subjected to electrophoretic fractionation at pH 8.6 in Longworth's barbiturate buffer by the present authors.

The barbiturate, rather than phosphate, buffer was employed since Haas (3) showed that phosphate interferes with the inhibition action. In control experiments we found barbiturate to be without this effect. The electrophoretic apparatus, the methods used for collection of the separate fractions, and the estimation of their protein content were the same as those employed in a previous investigation (6). The activity of the inhibitor was measured viscosimetrically following the details given in another report (7). Bovine testes were used as the source of hyaluronidase, and human umbilical cords as the source of the hyaluronic acid. The unit of hyaluronidase inhibition was taken as the ratio, $(R - R_0)/R_0$, where R_0 is the time in seconds required to reduce the viscosity of the reaction mixture (4.0 ml. buffered substrate soln. + 0.5 ml. buffered enzyme soln. + 1.5 ml. water) to half its original value, and R is the time in seconds required to reduce the initial viscosity to half its value after incubation (10 min. at 37.5°C.) of the buffered enzyme solution with 1.5 ml. of a 75-fold dilution of the serum with water prior to the addition of the buffered substrate.

From the results of two experiments, A and B, shown in the table it is apparent that, under the conditions employed, the hyaluronidase inhibitor activity migrated chiefly with the albumin fraction. This em-

TABLE I

Sample	Conc. of inhibitor in fraction			
	Units/ml.		Units/g. protein	
	A	B	A	B
Albumin	7.0	7.2	464	233
Albumin + α -globulin	6.7	4.9	268	229
Albumin + α - + β -globulin	4.8	—	161	—
α - + β - + γ -globulin	0.0	—	0	—
β - + γ -globulin	0.0	0.0	0	0
γ -globulin	0.0	0.0	0	0
Whole serum	4.3	9.8	62	151

phasizes the difference between the normal, ever-present, non-specific inhibitor in serum and the specific antibody inhibitors, which would be expected to occur in the γ -globulin. Further studies are being carried out on the chemical nature of the non-specific inhibitor.

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Book Review

A Text-book of Practical Organic Chemistry. By ARTHUR I. VOGEL. Longmans, Green, New York, N. Y., 1947. 1012 pp. Price \$10.50.

This volume is intended to give practising organic chemists the benefit of the author's long experience in research and teaching in the field of practical organic chemistry. The coverage is given by the following chapter headings: I, Theory of General Technique, 44 pages; II, Experimental Technique, 182 pages; III, Preparation and Reactions of Aliphatic Compounds, 258 pages; IV, Preparation and Reactions of Aromatic Compounds, 292 pages; V, Some Heterocyclic and Alicyclic Compounds, 12 pages; VI, Miscellaneous Reactions, 26 pages; VII, Organic Reagents in Inorganic and Organic Chemistry, 18 pages; VIII, Dyestuffs, Indicators, and Related Compounds, 12 pages; IX, Some Physiologically Active Compounds, 14 pages; X, Synthetic Polymers, 9 pages; XI, Qualitative Organic Analysis, 56 pages; Appendix, 38 pages.

Although there is quite a bit of useful information in this book I can muster no enthusiasm for it. Perhaps the most serious fault from the point of view of its practicality is the lack of suitable references. Quite a few important procedures are inadequately described and there are no references to review articles or even names of outstanding workers in the field to aid the reader in finding out more about such subjects. Specifically, I can mention material on dehydrogenation and on diazomethane although there are many other similar parts.

Another serious objection is the unevenness of presentation. Many pages are given over to relatively unimportant items or to repetition of examples of the preparation of similar types of compounds. At the same time some important information is lacking. For example, about 25 pages are devoted to the preparation of aliphatic halides from the corresponding alcohols and there is mention neither of the addition of hydrogen halides to unsaturated compounds nor of the fact that rearrangements frequently occur.

The description of laboratory apparatus is given in great detail in many places yet important improvements have been omitted. For example, the Hershberg melting point apparatus is not described and the method of preparing sodamide given in the text is inferior to that involving the use of liquid ammonia. Furthermore, the technique of centrifugation, and its many useful applications to practical organic chemistry, is not even mentioned.

Knowing the large amount of time and effort that is required to turn out a book, I would like to be able to recommend some part of the book highly so that it would take its place as a standard manual. Unfortunately, I cannot do this. There is too much material too unevenly presented to recommend for beginning students and it is too lacking in critical selection and too impractical for advanced students.

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Calcium Ion Exchanges in Some Normal Tissues and in Epidermal Carcinogenesis

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That the calcium content of malignant tissues is characteristically low has been well established. Experiments on this subject dating back to 1900 are thoroughly reviewed by Stern and Wilhelm (1). More recently, Brunschwig, Dunham and Nichols (2) and Suntzeff and Caruthers (3) confirmed the existence of a low calcium concentration in cancerous tissue.

Since there is an abundance of calcium available in the tissue fluids, the reduced amount of calcium in cancer cells must be due to decreased ability of calcium to enter the cells, or decreased ability on the part of the cancer cells to retain calcium. In an initial attempt to learn where and in what form calcium is lost from cancer cells, Lansing, Rosenthal and Au (4) conducted ultrafiltration studies on normal and hyperplastic epidermis and a transplantable squamous cell carcinoma. It was found that in hyperplastic epidermis (experimentally produced with methylcholanthrene) there was no alteration in the relative amounts of ultrafilterable and non-ultrafilterable calcium. The squamous cell carcinoma revealed a sharp reduction in ultrafilterable calcium. It was suggested that the base-binding capacity of an organic complex, probably a protein, is altered in this carcinoma. This view was extended to postulate a general relation between a calcium-binding complex and growth regulation by Lansing (5).

The current studies were undertaken to investigate the dynamics of calcium utilization in carcinogenesis. Considerable evidence has been accumulated by Heilbrunn and his associates (6) indicating that calcium is bound in the cortex of the cell. Mazia (7) has observed that, independent of the concentration of an ion outside the cell, this ion will not

be taken up by the cell unless it is first bound at the cell surface. It would be reasonable then to expect that, if the reduction of calcium-binding capacity observed in carcinoma is associated with the cell surface, malignant cells would be unable to take up the calcium ion. To test this possibility, radiocalcium (Ca^{45}) as traceable calcium was used in these experiments.

EXPERIMENTAL

The basic procedure employed in these studies involved quantitative measurement of radioactivity in epidermis as a function of time after injection. Ca^{45} was injected subcutaneously in the early experiments and intraperitoneally in the majority of experiments. No measurable difference in uptake of Ca^{45} using these routes of administration was detectable.

Isotope Technique

The stock isotope was radiocalcium (Ca^{45}) whose specific activity was 0.8 mc/25 g. CaCO_3 (as assayed by the Isotope Research Division, Atomic Energy Commission, Oak Ridge, Tenn., from whom the Ca^{45} was purchased). From this stock sample a standard injection solution of CaCl_2 was prepared containing approximately 2 mg./cc. solution (roughly 60,000 counts/minute).

Tissue samples containing the isotope were dry ashed in nickel crucible covers. A small quantity of dilute HCl was added to each dish to insure even distribution of the radioactive ash which was finally dried in a chemical drying oven at 110 C. Radioactivity counts were made with the Scott type thin mica window Geiger counter tube (Radiation Counter Laboratories) and with the Mark 2 Autoscaler (Tracerlab).

Calcium Analysis

Upon completion of the counting of radioactivity the ash in the nickel crucible covers was quantitatively transferred with dilute HCl to 15 ml. centrifuge tubes. The pH of the solution was adjusted to 5.2 with saturated sodium acetate, and calcium was precipitated as the oxalate using ammonium oxalate. The method of analysis was essentially that of Lindner and Kirk (8) which depends upon a back titration of ceric sulfate with Mohr's salt.

Biological Material

Three month old Swiss mice were used in the series on hyperplasia and 6-8 week old animals were used in the work with the transplantable squamous cell carcinoma. Hyperplasia was induced with 0.6% methylcholanthrene in benzene applied 3 times (every third day) in the series on 10 day hyperplasia, and applied 9 times in the series on 30 day hyperplasia. The backs of the animals were shaved 2-3 days prior to injection of 1 cc. of CaCl_2 solution containing the isotope. The animals were sacrificed at various intervals after injection by a sharp blow on the head. Blood, liver, bone (portions of the femur and tibia), and epidermis were removed for analysis. The epidermis was obtained by freezing whole skin with dry ice and scraping the epidermis free with a scalpel as the skin was thawing.

Mice 6-8 weeks old were used in the series on the squamous cell carcinoma. The tumor was obtained from the Barnard Free Skin and Cancer Hospital where it has been maintained by repeated transplantation for several years. The transplanted material was allowed to grow until each tumor was about 7-8 mm. in diameter, at which time the Ca^{45} solution was injected intraperitoneally. As in the series on hyperplastic epidermis, measurements were made of the Ca^{45} uptake and retention of blood, bone, liver, as well as the squamous cell carcinoma.

In all of the experiments the tissues of 5 mice were pooled to obtain each determination at the various time intervals illustrated in Figs. 1 and 2.

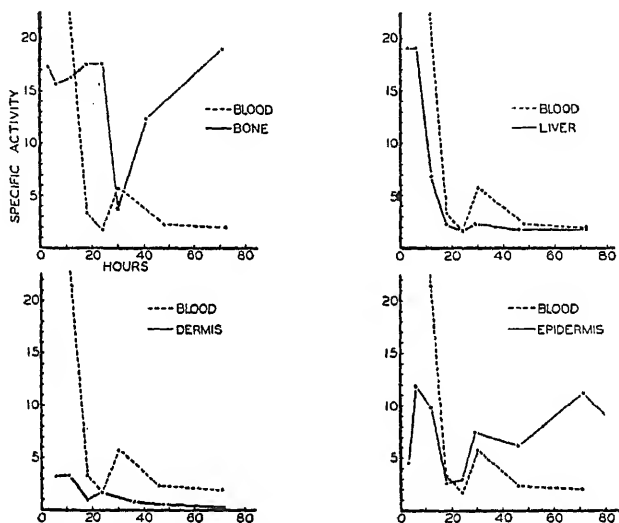


FIG. 1. Graphs contrasting specific activities of Ca^{45} in bone, liver, dermis, and epidermis of mice at various intervals after isotope injection. Each tissue is referred to the specific activity of blood. The data for bone have been uniformly reduced by a factor of ten.

RESULTS AND DISCUSSION

Normal Tissues

Fig. 1 is a graphic representation of the uptake and exchanges of radiocalcium in normal bone, liver, dermis, and epidermis as contrasted whole blood. The observed specific activities of bone at the various time intervals were extremely high and were reduced in the graph by a factor of 10 in order to make possible a visual contrast between the data for bone and the soft tissues.

Uptake of Ca^{45} by bone differs significantly from that of the soft

tissues. Radiocalcium in bone is retained at close to maximal level for 24 hrs., while, in the liver and dermis, the specific activity falls off abruptly soon after injection of the radiocalcium.

At 24 hrs. the specific activity of bone drops sharply, and at the same time that of blood increases significantly. Subsequently bone activity increases steadily to 72 hrs., while blood activity decreases to trace values. This sequence of events is illustrated graphically in Fig. 1. It

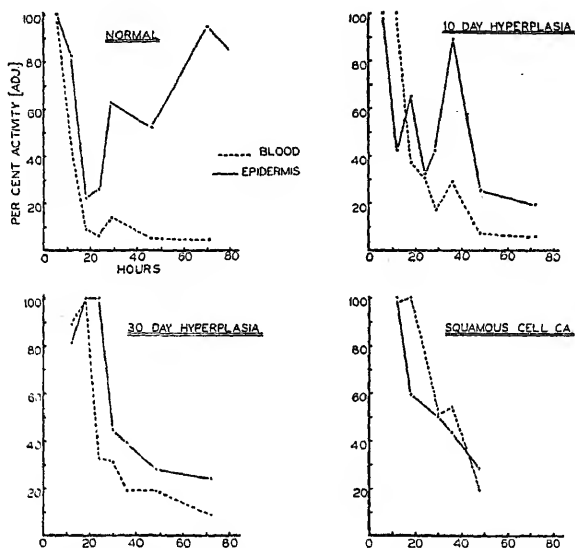


FIG. 2. Graphs showing changes in turnover of Ca^{45} in normal and hyperplastic epidermis, and in squamous cell carcinoma.

will be noted that the specific activities of bone have been uniformly reduced by a factor of ten in order to facilitate a comparison between it and blood. The transient drop in specific activity of bone at 24 hours after injection of Ca^{45} has been noted in all of our studies and is consistently associated with a transient increase in specific activity of blood. There is no indication that this rhythm is other than a result of the relatively high dose of calcium administered.

Both dermis and liver reveal a very low level of tagged calcium uptake indicating minimal calcium exchange. Liver reveals high specific activity levels shortly after injection, which probably result from the high level of radiocalcium in the blood and tissue fluids. Presumably

this excess calcium is removed from the fluids rapidly and the activity of the liver falls accordingly. Dermis, being a less vascular tissue than liver and therefore not containing as much blood, does not show this initial high specific activity.

Epidermis differs strikingly from the other soft tissues studied in that it actively takes up and exchanges tagged calcium at a level considerably above that of blood. This high level of activity persists through the 72 hr. period covered in these experiments.

Epidermal Carcinogenesis

Fig. 2 summarizes the data obtained from tagged calcium uptake experiments on normal and hyperplastic epidermis (10 and 30 day) and on the transplantable squamous cell carcinoma. The vertical axes of these graphs are based upon per cent activity adjusted to the calcium levels existing in each type of tissue. The epidermal calcium content of normal young Swiss mice is 44 γ /100 mg. of fresh tissue, that of hyperplastic epidermis is 19 γ /100 mg., and that of the squamous cell carcinoma is 9 γ /100 mg.

The 10 day hyperplastic series indicates a sequence of events through 36 hrs. which closely parallels the normal. After this time the per cent activity falls off abruptly.

The 30 day hyperplastic series establishes a new pattern of radio-calcium uptake. Twenty-four hrs. after injection the activity falls off and continues to decrease through 72 hrs. The activity curve for the hyperplastic curve parallels the blood curve rather closely.

Fewer time periods were studied in the transplantable squamous cell carcinoma series. However, it is clear that the radiocalcium activity of the tumor falls off steadily from 18 hrs. through 48 hrs.

The normal epidermis appears to be distinctly different from liver and dermis in that it turns over calcium actively. This property of epidermis is all the more striking since epidermis is an avascular tissue. Apparently then, the normal epidermal cell can take up from its inter-cellular fluids calcium which has diffused there from the blood. The observation that skin may have a relatively high exchange rate for calcium has been made before (9). This study goes further in that it indicates that it is only the epidermis which has the accentuated ability to exchange calcium. As shown in Fig. 1, the dermis does not possess any apparent capacity for calcium turnover.

The experiment on 10 day hyperplasia (3 applications of methylcholanthrene) seems to indicate that, while this hyperplastic epidermis is still able to actively store radiocalcium, at least through 36 hrs. after injection, it is not able to retain this stored radiocalcium. In this respect it differs from the normal.

The 30 day hyperplastic series (9 applications of methylcholanthrene) indicates a complete change from the pattern of radiocalcium exchange. This hyperplastic epidermis shows no ability to take up calcium and the amount of radiocalcium in it is determined by the concentration of the isotope in the blood. Thus, the activity curves for blood and 30 day hyperplastic epidermis are essentially parallel. This same sequence of events also exists in the radiocalcium uptake of the squamous cell carcinoma. We have concluded that, during methylcholanthrene-induced carcinoma, there is a more or less progressive loss of the ability of epidermis to turn over calcium.

As mentioned in the introductory section of this paper, Mazia (7) has suggested that the uptake of an ion by a cell is dependent upon binding of that ion at the cell surface. Presumably, an alteration in the ability of the cell surface to bind the ion would be reflected in an altered uptake of the ion by the cell. Our present study indicates that the squamous cell carcinoma as well as the methylcholanthrene-induced late hyperplasia are characterized by an abnormally low exchange of calcium ions. This observation supports the view expressed earlier (5) that cancer involves an alteration in a calcium-binding (protein) complex which may be associated with the cell surface. This finding is also consistent with the observation (4) that the base-binding capacity of the complex is altered in carcinoma.

SUMMARY

The radiocalcium turnover of normal bone, blood, epidermis, dermis, and liver, as well as that of experimentally induced hyperplasia and squamous cell carcinoma have been studied.

These experiments indicate that epidermis, unlike other soft tissues, actively exchanges calcium. In early methylcholanthrene-induced hyperplasia there is a loss of storage capacity in epidermis for this calcium. Thirty day hyperplastic epidermis and a transplantable squamous cell carcinoma are characterized by an inability to exchange calcium ions.

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Amino Acids as Nitrogen Source for the Growth of Yeasts

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INTRODUCTION

Although the sources of nitrogen which may be utilized in growing yeast are numerous, the literature contains only meager information concerning the influence of different nitrogen sources upon the growth rate of specific yeast cultures. Studies on the availability of the nitrogen of amino acids for the growth of yeast have been reported by Thorne (1), Nielsen (2,3,4) and Sperber (5), but their data have been limited to comparatively few yeast strains. It has been shown by Mitchell and Williams (6), Atkin, Schultz and Frey (7), and Hertz (8) that, in a basal medium containing known growth-promoting factors, many yeasts grow faster when the sole source of nitrogen is acid-hydrolyzed vitamin-free casein than when nitrogen is supplied as ammonium sulfate. (Hence, casein hydrolyzate was used in the determinations of B-complex vitamins by yeast microbiological methods, and in the bios investigation (9) reported from this laboratory.) The purpose of the present study is to correlate and extend the above work by determining the adequacy of individual amino acids as sources of nitrogen for the growth of each of a number of strains of yeast in basal media of known composition. Casein hydrolyzate and ammonium sulfate are used as the organic and inorganic nitrogen controls. The data reported may be valuable in further characterization of yeasts, and may possibly aid in the elucidation of the mechanism of deamination and protein synthesis.

EXPERIMENTAL

The procedure used was essentially that described by Atkin, Schultz and Frey (7), and Schultz and Atkin (9). The latter paper contains a detailed account of the preparation of the various solutions used to prepare the basal medium, as well as the sources of the yeast cultures, the preparation of the yeast inoculum and the method for

measuring yeast growth. However, instead of employing a single omission technique of the growth factors, as was done in the previous study (9), the present work kept the growth factors in the basal medium constant, and substituted individual amino acids for the nitrogen controls.

Basal Medium

To determine the nitrogen requirements of 5 yeast cultures at a time, using 22 different nitrogen sources, make up the basal medium with the following proportions:

Sugar and salts (SAS) solution	280 ml.
Potassium citrate buffer	56 ml.

Prepare a solution of the B-complex vitamins by mixing 56 ml. portions of each of the following compounds in the concentrations indicated:

Inositol	500 γ /ml.
Calcium pantothenate	200 γ /ml.
Biotin	100 m γ /ml.
Thiamine hydrochloride (B_1)	10 γ /ml.
Pyridoxine hydrochloride (B_6)	10 γ /ml.
Nicotinic acid	10 γ /ml.

Arrange in racks, numbered test tubes (18×150 mm.) calibrated for absorption (7). Twenty-two test tubes are needed for each yeast culture. To each tube, add 3 ml. of the basal medium, 3 ml. of the vitamin solution, and 3.5 ml. of a distilled water solution of one of the nitrogen sources listed in the typical protocol (Table I). Wherever the use of U.S.P. casein as a nitrogen control is indicated in the tabulated data, the casein is added as an acid-hydrolyzed casein solution (9).

The amino acid solutions used for this work can be made up in 50 or 100 ml. lots, sterilized in flowing steam for 30 min., and then stored at $4-7^\circ\text{C}$. until used. It may be necessary to heat some of these solutions before use in order to dissolve material which settles out during storage.

The amount of available nitrogen to be added to a single tube in the case of each nitrogen source is listed in Table I. (These amounts were determined to be approximately optimum by running concentration response curves with each nitrogen source, except where low solubility precluded use of an optimum amount, as in the case of cystine.) As can be seen from Table I, the amounts generally approximate 4 mg. of available nitrogen per tube. This amount is not very critical, since in many instances 2 mg. will give essentially the same crop. In the absence of analytical data on the protein content of the yeast, however, the higher amount is considered preferable.

Yeast Inoculum

The preparation of the yeast inoculum used in this study has been described previously (9). The inoculation per tube consists of 0.5 ml. of sterile saline solution containing approximately 0.07 mg. moist yeast.

Yeast Growth

The yeasts are grown at 30°C. with shaking, and the extent of growth is measured at 16, 20, 24, and 40 hr. intervals of growth by reading percentage absorption of light on a Lumetron 400 colorimeter fitted for use with 18 mm. O.D. test tubes. The white light used in the Lumetron is suitably reduced in intensity by a filter made of a gray glass and wire screen combination.

TABLE I
*Typical Protocol: Individual Amino Acids Used as the
 Only Nitrogen Source for Yeast Growth*
 (Yeast culture: *Saccharomyces cerevisiae* Hansen, strain *anamensis*)

Nitrogen source	Available nitrogen ^a per test tube	Per cent absorption			
		After 16 hr.	After 20 hr.	After 24 hr.	After 40 hr.
	(mg.)				
Reference controls:					
Ammonium sulfate	3.96	63	91	93	94
U.S.P. casein	3.87	85	92	92	94
Amino acids:					
DL-Alanine	4.72	7	30	61	92
L-Arginine·HCl	3.99 ^d	57	92	92	94
L-Asparagine	4.00 ^b	48	91	93	94
DL-Aspartic acid	4.00	8	40	81	93
L-Cystine	0.12 ^c	0	0	0	1
L [+]-Glutamic acid	4.00	5	38	86	94
Glycine	4.14	0	0	0	0
L-Histidine·HCl	3.99 ^b	0	0	1	2
DL-Isoleucine	0.50	3	8	23	80
L [-]-Leucine	4.02	20	62	83	88
L [+]-Lysine·HCl	3.99 ^b	0	0	0	0
DL-methionine	4.00	2	5	16	83
DL-Phenylalanine	3.48	11	37	74	91
L-Proline	7.30	10	31	64	94
DL-Serine	1.00	3	17	43	82
DL-Threonine	3.99	0	3	0	3
L [-]-Tryptophan	2.33 ^b	5	13	24	83
L-Tyrosine	0.23	4	17	40	63
Urea	3.99 ^c	30	80	92	94
DL-Valine	3.99	11	43	82	90

^a Calculations based on L (natural) isomers only.

^b Calculations based on one utilizable nitrogen.

^c Calculations based on two utilizable nitrogens.

^d Calculations based on four utilizable nitrogens.

TABLE II

Adequacy of Individual Amino Acids as the Only Nitrogen Source for Growth of 40 Saccharomyces Yeasts

Nitrogen source	Individual <i>Saccharomyces</i> yeasts		
	Good growth	Medium growth	Poor growth
Reference Controls:			
Ammonium sulfate	39	0	1
U.S.P. casein	39	0	1
Adequate:			
Alanine	40	0	0
Arginine	40	0	0
Asparagine	40	0	0
Aspartic acid	40	0	0
Glutamic acid	40	0	0
Leucine	40	0	0
Urea	40	0	0
Valine	40	0	0
Inadequate:			
Cystine	0	0	40
Glycine	2	0	38
Histidine	1	0	39
Lysine	1	0	39
Threonine	0	0	40
Variant:			
Isoleucine	7	25	8
Methionine	16	20	4
Phenylalanine	23	14	3
Proline	25	8	7
Serine	23	15	2
Tryptophan	9	27	4
Tyrosine	2	31	7

Growth Evaluation

In evaluating the growth of some of these yeasts after 40 hr. incubation at 30°C. with shaking, absorption values falling between 0-20% are scored as "poor," those between 20-70% as "medium," and those between 70% and full growth as "good." An end reading of 0% corresponds to no multiplication (0.007 mg. moist yeast/ml.), a reading of 20% to a 100-fold multiplication (0.7 mg. moist yeast/ml.), and a reading of 70% to a 570-fold multiplication (4.0 mg. moist yeast/ml.). Full growth is attained at a concentration of about 25 mg. moist yeast/ml., a multiplication of 3500-fold or more.

RESULTS

Yeasts comprising a number of genera have been tested for their ability to utilize the nitrogen of individual amino acids as the only source of nitrogen.

The protocol in Table I illustrates the generally slower growth rate of *Saccharomyces* yeasts on amino acids as compared with growth on casein or ammonium sulfate. However, marked acceleration of the growth rates of many *Saccharomyces* yeasts on certain of the amino acids is evidenced between the 24-hr. and 40-hr. growth periods.

After an extensive evaluation of approximately 40 species, strains, and varieties of *Saccharomyces* yeasts (Table II), the following generalizations can be made, keeping in mind the experimental conditions under which the yeasts were tested: 1. Yeasts of the genus *Saccharomyces* produce good crops on alanine, arginine, asparagine, aspartic acid, glutamic acid, leucine, urea, and valine, but, on the whole are unable to utilize the nitrogen from cystine, glycine, histidine, lysine, and threonine. 2. Moreover, certain amino acids support good growth with certain yeasts of the genus *Saccharomyces*, and only poor or intermediate growth with other yeasts of this genus. In this category are the following amino acids: isoleucine, methionine, phenylalanine, proline, serine, tryptophan, and tyrosine. When fully substantiated, these differences in nitrogen utilization may serve as a further aid in the classification or subdivision of the otherwise confusingly similar group of organisms belonging to the genus *Saccharomyces*. Differences in the bios requirements of these yeasts have already been demonstrated (9).

Yeasts of the genus *Torula* (*Torulopsis*) show more diversity than *Saccharomyces* yeasts in their ability to utilize the nitrogen from amino acids (Fig. 1). A greater proportion of the *Torula* yeasts tested attained good growth on glycine, lysine, and methionine than did the *Saccharomyces*. The two species *dattila* and *stellata* showed markedly poorer growth rates than did the other *Torula* yeasts.

Both of the *Candida* yeasts included in Fig. 1. were remarkably vigorous in their growth on individual amino acids. *C. guilliermondi* showed full growth on all the amino acids used, except cystine, histidine, and tyrosine, on each of which it attained medium growth. *C. pseudotropicalis* was unable to use cystine, showed medium growth on glycine and tyrosine, and full growth on all others.

The two *Hansenula* yeasts grew only poorly on cystine, histidine, lysine, and threonine. The remaining yeasts in Fig. 1 (3 *Pichia*, 3 *Zygosaccharomyces*, and 1 *Klöckera*) showed generally good growth on all the amino acids, with the exception of cystine, glycine, histidine, threonine, and tyrosine.

NITROGEN SOURCE:																		
	Ammonium sulfate	U.S.P casein	Alanine	Arginine	Asparagine	Aspartic acid	Cystine	Glutamic acid	Glycine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenyl alanine	Proline	Serine	Threonine
CULTURE																		
<i>Torula pulcherrima</i>	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■
<i>Torula utilis</i>	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■
<i>Torula thermophilum</i>	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■
<i>Torula cremoris</i>	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■
<i>Torula colliculosa</i>	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■
<i>Torulopsis fermentati</i>	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■
<i>Torulopsis dothidea</i>	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■
<i>Torulopsis stellata</i>	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■
<i>Candida guilliermondii</i>	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
<i>Candida pseudotropicalis</i>	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■
<i>Hansenula lambica</i>	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■
<i>Hansenula anomala</i> var <i>robusta</i>	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■
<i>Pichia belgica</i>	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■
<i>Pichia mandshurica</i>	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■
<i>Pichia kluyveri</i>	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■
<i>Zygosaccharomyces lactis</i>	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■
<i>Z. morixianus</i>	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■
<i>Z. prioranus</i>	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■
<i>Klöckera brevis</i>	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■

40 HOUR GROWTH:	
Good (70-95% light absorption) =	■
Medium (20-70% light absorption) =	▨
Poor (0-20% light absorption) =	□

FIG. 1. Adequacy of individual amino acids as the only nitrogen source for the growth of yeast cultures of various genera.

DISCUSSION

Under the conditions of our experiments, the principal contribution made by amino acids to the growth of yeast was to supply assimilable nitrogen. Among the amino acids investigated, many did not support as rapid growth of any individual culture as did ammonia nitrogen. A few amino acids, on the other hand, supported more rapid growth than did ammonia nitrogen. These differences in growth rates are probably due to the marked differences in structural configurations of the amino acids and to variations in the ease of deamination. On the other hand, differences of growth response of various cultures with a given amino acid may be attributable to differences in their respiratory enzyme systems. The elucidation of the mechanism by which casein hydrolyzate, a multiple source of nitrogen, supports a faster rate of yeast growth than does ammonia nitrogen or any single amino acid requires further investigation.

Since it is known (10,11) that the amounts of growth-promoting factors in the media influence the nitrogen assimilation of yeast, a supposedly ample supply of all growth-promoting factors was included in the basal media employed for these studies. Further experimentation along these lines might reveal specific bios factor combinations which would improve the assimilation of nitrogen from different amino acids.

It is possible that differences in growth responses of yeasts to individual amino acids might be used as a basis for classifying yeast cultures. Such a method might be used advantageously as a supplement to the system of classification which makes use of bios requirements (9). Not enough cultures have been studied to make a complete formulation, but certain characteristics are evident. For instance, most *Saccharomyces*, particularly *S. cerevisiae* Hansen yeasts, are unable to use cystine, glycine, histidine, lysine, and threonine under the conditions of the test. Furthermore, these yeasts, with few exceptions, can grow well on alanine, arginine, asparagine, aspartic acid, glutamic acid, leucine, urea, and valine. Conversely, some yeasts in genera such as *Torula*, *Candida*, and *Pichia*, are able to utilize glycine and lysine to varying degrees but are unable to make use of certain of the other amino acids.

Several yeasts demonstrated accelerated growth rates with a few of the amino acids, usually between the 24 and 40 hr. growth periods. In

such cases, adaptation is suspected. Further study of this phenomenon may prove enlightening.

The amino acids which Thorne (1) rated as "good" or "bad" nutrients for a top fermentation brewery yeast were similarly rated for the *Saccharomyces* yeasts listed in Table II. However, there are many amino acids in Thorne's "average" group which we found to give either "good" or "poor" response with a number of strains of *Saccharomyces* yeasts.

Nielsen's experiments (2) on the nitrogen assimilation by brewery bottom yeast demonstrated that glycine and histidine were fully utilized. Our results are at variance with Nielsen's, possibly because he made use of experimental conditions which confined his yeast to a small number of multiplications over a long period of time.

Sperber (5) limited his work along these lines mostly to *Torula utilis* and to a few amino acids. His results showed that α -alanine, asparagine, and DL-aspartic acid were readily utilized, while glycine and β -alanine were less readily utilized under the experimental conditions employed.

Torula yeasts have been shown herein to utilize the nitrogen from a greater number of amino acids than have *Saccharomyces* yeasts. According to Nielsen (4), haploid yeasts are less capable of assimilating nitrogen from beer wort than are diploid yeasts. Therefore, it is possible that the contention of Lindegren (12), Winge (13), and Satava (14) that *Torula* yeasts are haploid forms derived from the genus *Saccharomyces* is false.

SUMMARY

Yeasts comprising a number of genera and species have been studied with respect to their ability to use individual amino acids as the only source of nitrogen. General trends in the utilization of the nitrogen of amino acids were noted with different strains and varieties of yeast of the same genus, but some differences were also apparent. The value to yeast classification of different growth responses on individual amino acids has been indicated.

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Metal Antagonism of the Antibacterial Action of Atabrine and Other Drugs

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INTRODUCTION

It has been shown that the activity of a sample of enzymatically digested lactalbumin in antagonizing the growth inhibition of *Escherichia coli* by atabrine could be accounted for by its calcium and magnesium content (1). This antagonism has been investigated further, and it has been found that the metals active in the phenomenon are Ca^{++} , Mg^{++} , Mn^{++} , and Ba^{++} ; that a stoichiometric relationship holds between atabrine and Ca^{++} in their effects on *E. coli* growth; that citrate intensifies the bacteriostatic effects of atabrine; and the calcium reversal effects hold for antimalarials other than atabrine in the drug-induced bacteriostasis of *E. coli*.

MATERIALS AND METHODS

The test conditions were essentially those described previously (2). The basal medium employed consisted of 1.0% Bacto-peptone, 0.6% NaCl, and 0.2% glucose. Bacto-peptone lot number 314990 was used throughout. It was selected because of its low content of antagonists against atabrine and contained 0.20 γ Ca and 0.60 γ Mg/mg.¹

The organisms employed were *E. coli* ATCC number 9723 and *Torula utilis* ATCC number 9255.

Inorganic salts employed were Mallinckrodt reagent grade. All antimalarials were obtained from the Division of Tropical Diseases, Malaria Subdivision, the National Institute of Health; the cyanine dye was the gift of Parke, Davis and Co. and the Eastman Kodak Co.

The Effect of Cations

Of the metallic salts (as chlorides) tested for their anti-atabrine activity, only 4 were effective. They are Ca^{++} , Mg^{++} , Mn^{++} , and Ba^{++} . Their relative activity is in-

¹ I am indebted to Mr. Charles Kinser for this analysis.

TABLE I
The Antagonism of Atabrine Action by Cations

Cation	Concentration, γ /tube				
	0	20	60	120	180
	hrs.	hrs.	hrs.	hrs.	hrs.
Ca ⁺⁺	> 48	19	5.0	4.5	4.5
Mg ⁺⁺	> 48	22.0	5.75	5.0	4.75
Mn ⁺⁺	> 48	> 48	6.75	6.25	5.75
Ba ⁺⁺	> 48	> 48	> 48	6.75	6.75

Values indicate time in hours required for appearance of visible turbidity.

In absence of atabrine visible turbidity was evident in 4.5 hrs.

Atabrine concentration: $1\mu M$ in final volume of 5 ml. Initial pH: 7.45. Inoculum: 1 drop 24 hr. growth of *E. coli* diluted 1/1000.

indicated in Table I. The following, as chlorides, when tested at concentrations below their toxic levels, did not antagonize the inhibitory effects of atabrine: Cu⁺⁺, Zn⁺⁺, Fe⁺⁺, Co⁺⁺, and Ni⁺⁺.

The Effect of Sodium Citrate

MacLeod and Snell (3) have demonstrated that mineral deficiencies for several lactobacilli could be produced in media by the addition of sodium citrate. Growth

TABLE II
The Combined Effect of Sodium Citrate and Atabrine

Citrate conc. per cent	Atabrine conc. μM /tube				
	0	0.2	0.4	0.6	0.8
	hrs.	hrs.	hrs.	hrs.	hrs.
Exp. A					
0	4.5	5.25	12	18	> 96
0.2	5.0	28	> 96	> 96	
1.0	5.25	33	> 96	> 96	
2.0	5.75	> 96	> 96	> 96	
Exp. B					
0	4.25	4.75	5.0	7.0	
.04	4.5	4.75	6.25	20	
.08	4.5	4.75	6.75	> 48	
.16	4.75	5.0	16.0	> 48	

Initial pH Exp. B, 7.25. Other conditions indicated in Table I. Volume: 5 ml.

inhibition could be prevented by the addition of Mg^{++} , Mn^{++} , or Ca^{++} . Thus, it would be expected that the presence of sodium citrate would enhance the inhibitory effect of atabrine in the growth of *E. coli*. The results are shown in Table II. With suitable concentrations of sodium citrate, effective bacteriostasis can be obtained at low atabrine concentrations. The effect of the citrate is evident at concentrations as low as 0.04%.

Relationship between Atabrine and Ca^{++} Concentrations

There appears to be a stoichiometric relationship between atabrine concentration and the concentration of $CaCl_2$ required to antagonize completely the inhibitory effect. This relationship was established by the procedure indicated in Table III. The reversal ratios (Ca^{++}/AT) were established by selecting the lowest concentrations of $CaCl_2$ which completely reversed the action of a fixed concentration of atabrine.

The low ratios found at the lower level of atabrine concentrations are due in part to the Ca-Mg content of the basal medium. In the volume of medium employed (5 ml.), the 50 mg. of bacto-peptone present contributed 10 γ Ca and 30 γ Mg, the combined activity of which is equivalent to about 1 μM Ca^{++} . If this Ca-Mg addition is considered, the Ca^{++}/AT ratios would in all cases be in the region of 4. Thus, it is apparent a

TABLE III.
Calcium Reversal with Increasing Atabrine Concentrations

$CaCl_2$, micromoles		Reversal ratios, Ca^{++}/AT
	Atabrine, 0 micromoles	
0	4.5hrs.	
8	4.5	
16	4.5	
	Atabrine 0.5 micromoles	
0	5.0 hrs.	
.25	4.75	
.5	4.75	
1.0	4.5	2
2.0	4.5	
	Atabrine 0.75 micromoles	
0	18 hrs.	
.375	5.25 hrs.	
.75	4.75	
1.5	4.5	2
3.0	4.5	
	Atabrine 1.0 micromoles	
0	> 48 hrs.	
.5	11	
1.0	5.25	
2.0	4.75	
4.0	4.5	4

TABLE III (continued)

CaCl ₂ , micromoles		Reversal ratios, Ca ⁺⁺ /AT
Atabrine 1.5 micromoles		
0	> 48 hrs.	
.75	27	
1.5	7.5	
3.0	4.75	
6.0	4.5	4
12.0	4.5	
Atabrine 2.0 micromoles		
0	> 48 hrs.	
1	> 48	
2	12	
4	4.75	
8	4.5	4
12	4.5	
Atabrine 2.5 micromoles		
0	> 48 hrs.	
1.25	> 48	
2.5	15	
5.0	5.0	
10.0	4.5	4
15.0	4.5	
Atabrine 3.0 micromoles		
0	> 48 hrs.	
1.5	> 48	
3.0	18	
6.0	5.0	
12.0	4.5	4
18.0	4.5	

Experimental conditions as in Table I. Volume: 5 ml.

stoichiometric relation exists between the inhibitory concentrations of atabrine and the amount of Ca⁺⁺ required to relieve this inhibition.

In determining drug/antagonist ratios, it must be borne in mind that some variation is to be expected due not only to errors inherent in the assay procedure, but also to slight variations in drug sensitivity of the test organism and, in the case of atabrine, slight variations in the pH of the medium.

Ca⁺⁺ Reversal of Other Drugs

In addition to its effects on atabrine bacteriostasis, CaCl₂ in suitable concentrations will also reverse the action of other drugs. Table IV lists the concentration of the drug required to prevent the appearance of visible growth for 24 hrs. or more. Also indicated is the molar ratio, Ca⁺⁺/drug, required to bring about complete reversal in the growth *E. coli*.

Plasmoquin is a poor bacteriostatic agent for *E. coli* and since it was employed as the citrate, the Ca^{++} /plasmoquin ratio indicated the Ca^{++} required to overcome the combined effects of citrate and plasmoquin. Thus, the true Ca^{++} /plasmoquin ratio would be lower than that indicated.

The cyanine dye included in the table is a member of the dye series shown to have pronounced antifilarial activity (4). Its action is striking in that it is an effective bacteriostatic agent at low concentrations and large amounts of Ca^{++} are required to reverse its action.

TABLE IV
Reversal of Drug Effects by Calcium Chloride

Drug	conc. inhibiting 24 hrs. micromoles in 5 ml. vol.	Range of reversal ratios, Ca^{++} /drug
Atabrine. 2HCl	1	3—6
Quinine. 2HCl	5	0.4—0.6
Paludrine. HCl	2.6	8—12
Plasmoquin citrate	8.8	0.3—0.5
Pentaquine monophosphate	5	0.3—0.4
1, 1 ¹ —dimethyl—2, 2 ¹ — cyanine chloride	0.35	40—60

The ratios indicated in Table IV are based on the added Ca^{++} concentrations, ignoring the Ca-Mg content of the basal medium. The Ca^{++} /drug antagonism cannot be demonstrated successfully in a mineral salts-glucose medium which nonetheless supports the growth of *E. coli*. Apparently bacto-peptone contains a factor which is necessary for the demonstration of this phenomenon.

Atabrine Inhibition of Torula utilis

Although the atabrine bacteriostasis of *E. coli* may be reversed by the addition of suitable concentrations of Ca^{++} , *T. utilis* does not respond in a similar fashion. *T. utilis* resembles *E. coli* in that it grows readily in a mineral salts-glucose medium. In bacto-peptone medium, *T. utilis* growth is inhibited by $0.2 \mu\text{M}$ atabrine in a final volume of 5 ml. The addition of $20 \mu\text{M}$ CaCl_2 , yielding a Ca^{++} /AT ratio of 100, does not result in a relief of this inhibition.

DISCUSSION

There have been several reports which indicate that a calcium-anti-malarial relationship exists. However, the results reported for *E. coli* represent a case in which this relationship has been established for a microorganism.

In 1944, Keogh and Shaw (5) reported that the addition of Ca^{++} to isolated intestine previously treated with quinine or other antimalarials induces contraction rather than the normal response of relaxation, an effect comparable to that of calcium deficiency obtained by other

means. Williamson *et al* (6), have shown that high calcium diets significantly increased toxicity of atabrine in rats and resulted in higher concentrations of atabrine in the liver and spleen. These results, together with the data reported in this paper, indicate a relationship between calcium and atabrine in biological systems. Its nature is not yet apparent. However, it is of some interest that the Ca^{++} /drug ratios for quinine, atabrine and paludrine roughly parallel the relative activities of these compounds in suppressing blood-induced infections of *Plasmodium lophurae* in the duck (7).

SUMMARY

1. In descending order of activity Ca^{++} , Mg^{++} , Mn^{++} , and Ba^{++} antagonize the bacteriostatic effects of atabrine in the growth of *E. coli*.
2. Sodium citrate greatly intensifies the inhibiting effect of atabrine.
3. There is a stoichiometric relationship between atabrine concentration and the concentration of Ca^{++} required to relieve the atabrine inhibition.
4. In addition to atabrine, the inhibiting effect on *E. coli* of the drugs quinine, paludrine, plasmoquin, pentaquine, and a cyanine dye are successfully antagonized by Ca^{++} .

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NOTE ADDED ON PROOFING

Recently, evidence has been presented [MASSART, L., PETERS, G., VAN HOUKE, A., AND LAGRAIN, A., *Arch. intern. pharmacodynamie* **75**, 141 (1947)] that cations compete with acridines for adsorption on cellular ribonucleoproteins.

Antifungal Activity of Hop Resin Constituents and a New Method for Isolation of Lupulon

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INTRODUCTION

Mature hops contain 11–21% of resin containing two antibiotic compounds known as humulon and lupulon (1,2,3,4). Hops impart to beer wort antibiotic activity against certain Gram-positive bacteria but not against certain Gram-negative bacteria (4). According to reviews by Wallerstein (1) and Walker (2), humulon, lupulon, and other constituents of hop resin are responsible for this antibiotic activity. This paper describes the antibiotic activity of hop resin and its constituents against fungi, and a new method for the preparation of lupulon.

PREPARATION OF MATERIALS

Hop resin was fractionated by a scheme (Fig. 1) based on the method described by Rabak (5).

Soft Resin

Hops (1025 g.), stored cold since drying, and ground without heating, were extracted first with 6.6 l., and then with 2.7 l., of technical methanol. The combined extracts, totaling 9 l., were added to 15 l. of 2% NaCl. The resulting mixture was extracted successively with five 5-l. portions of 30–60° petroleum ether. The combined petroleum ether extracts were evaporated under reduced pressure until the soft resin remained as a very viscous dark brown liquid (yield, 123 g.).

Hard Resin

A portion of the methanol-water phase, containing the hard resin, was evaporated to dryness. As this material contained negligible antifungal activity, further purification of the hard resin was not attempted.

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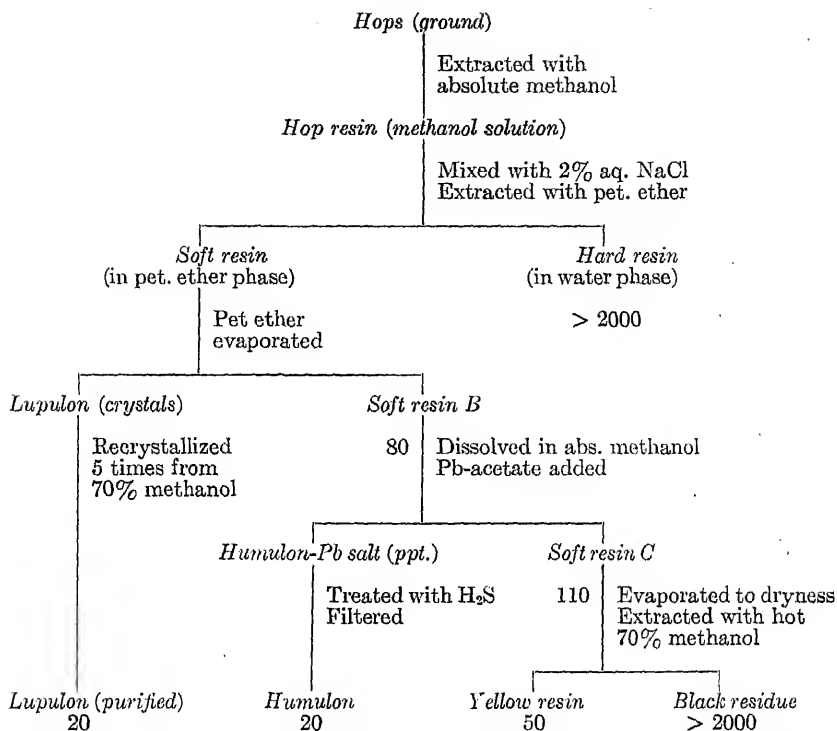


FIG. 1. Scheme for fractionation of hop resin. The figure under each fraction is the concentration (mg./l.) giving 50% inhibition of mycelial growth of *Sclerotinia fructicola*.

Lupulon

A new method was developed for the isolation of lupulon. After storage overnight at 2°C., the soft resin contained lupulon crystals. The mixture was warmed to room temperature, mixed with 50 ml. of petroleum ether to reduce its viscosity, and filtered through a fritted glass filter. Thirty-nine g. of lupulon crystals and adhering resinous material were obtained. A sample of this material deteriorated on prolonged storage at 2°C.

Eight g. of the crude lupulon were refluxed briefly with shaking in 320 ml. of 70% methanol. The solution was decanted and filtered while hot, leaving about 2 g. of insoluble impurities. When the filtrate was cooled slowly to 2°C. and held overnight at this temperature, large

crystals separated. The supernatant, containing small particles of amorphous material, was decanted. The yield of 2.25 g. of colorless lupulon crystals contained only a trace of resinous material. About 1 g. of mixed crystals and resinous material from the bottom of the container was rejected. To effect this separation, the concentration of methanol is critical. It could not be made with 60 or 80% methanol.

The lupulon was crystallized 4 times from hot 70% methanol, using about 60 ml. of solvent/g. of lupulon. It was dissolved by refluxing, with shaking, for 2-5 min. A small amount of insoluble resinous material, which was thought to be a decomposition product of lupulon, frequently appeared during this process and was separated mechanically. The solution was then cooled slowly to 2°C., and held overnight.

The final yield of recrystallized lupulon was 1.34 g. Comparable yields were obtained from other runs. It melted at 88-92°C., and had no optical rotation in benzene solution. Lupulon is described as optically inactive (6), and as melting at 90.5-92°C. (7). Humulon, the only other crystalline material reported from hop resin, has a m.p. of 65-66.5°C and $[\alpha]_D^{20}$ of -232° in benzene (8). Purified lupulon was tasteless and retained its appearance and biological activity during several months of storage in air at 2°C.

Analysis. Theoretical: C, 75.3%; H, 9.24%. Found: C, 74.3%; H, 9.02%.²

Humulon

Using a modification of Rabak's procedure (5), a 12 g. portion of soft resin B (Fig. 1) was dissolved in 400 ml. of absolute methanol. Humulon was precipitated by the addition of lead acetate.³ The precipitate was suspended in ethyl ether and treated with H₂S (9). After filtration, the solvent was evaporated under reduced pressure, leaving a light brown residue. This was dissolved in methanol, reprecipitated with lead acetate, and recovered in the same way. The resulting light yellow residue (2.0 g.) crystallized on standing in the cold. The product obtained in this manner consists almost entirely of humulon (10) and is almost as active against *Lactobacillus bulgaricus* as is highly purified humulon (9).

Other Fractions of Soft Resin

After precipitation of the humulon, the supernatant contained a slight excess of lead which was precipitated as PbS. The filtrate was evaporated, leaving a dark

² Mr. Gordon Alderton of this laboratory prepared lupulon from lupulin (naturally occurring hop resin) by this method and obtained the following analysis: C, 75.0%; H, 9.10%.

³ Later observations indicate that under these conditions, use of a considerable excess of lead acetate gives a higher yield of humulon than is reported here.

viscous residue (soft resin C (Fig. 1)). This was extracted with 250 ml. of hot 70% methanol, yielding 3.3 g. of yellowish brown viscous material which was termed "yellow resin." Two subsequent extractions yielded a total of 1.2 g. of similar material which was of darker color and lower in antibiotic potency. The next two extractions yielded only 0.2 g. of material; 1.1 g. of black residue remained.

Humulon and lupulon are both soluble in hot 70% methanol. Any of these substances not separated from the soft resin in the previous steps would, therefore, have been recovered as part of the "yellow resin."

Storage of Preparations

The preparations were stored at 2°C. as a precaution against deterioration (1,2), and were freshly dispersed for bioassays.

BIOASSAY METHODS

Preparation of Materials for Bioassay

Since these preparations (except hard resin) are relatively insoluble in water, they were dispersed as follows: Soft resin was dispersed in water containing 0.2% agar by means of a portable hand-operated homogenizer. The resulting emulsion was reasonably stable and could be added to the nutrient medium for assay. This method was not satisfactory for the other preparations. Humulon, yellow resin, and soft resins B and C (Fig. 1) in 40 mg. quantities were dissolved or dispersed in 2 ml. of warm ethylene glycol, to which was added 100 ml. of hot water. Lupulon and the "black residue" were dispersed similarly, except that the 2 ml. of ethylene glycol was replaced by 0.15 ml. of "Tween 20" (sorbitan monolaurate—polyoxyalkylene derivative, an emulsifying agent marketed by the Atlas Powder Co.).⁴ When the amount of "Tween 20" was increased, both the turbidity and the antifungal activity of the preparation were reduced.

Immediately after preparation, these dispersions were adjusted to pH 5.8 (at which they were to be assayed) and added to the assay medium as described below. They were not sterilized, because hop antibiotics are known to be heat labile under some conditions (2). Contaminants appeared only occasionally.

Bioassay Using Mycelial Growth

Hop antibiotics were added in various concentrations to potato dextrose agar medium,⁵ from which Petri plates were poured in duplicate. Each plate was then

⁴ The mention of this product does not imply that it is endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.

⁵ The potato infusion medium was prepared as follows: 250 g. of diced potato/l. of distilled water was heated for 1 hr., strained through cheesecloth, and autoclaved for 30 min. 2% glucose and 1.5% agar were added and the pH was adjusted to 5.8. This medium was slightly cloudy. Growth of some fungi was better than on Difco Potato Dextrose Agar.⁴

inoculated in the center with agar plaques cut with a sterile cork borer from near the margin of rapidly growing colonies of the test fungus.

After incubation, the diameter of each colony was used as the measure of growth. The percentage of maximal growth was plotted against the concentration on logarithmic-probability paper (11) as shown in Fig. 2. From the resulting dosage-response curve, the concentration which would have given 50% inhibition of growth was determined, and this was taken as a measure of the potency of the preparation. A discussion of antifungal bioassays is planned elsewhere (12).

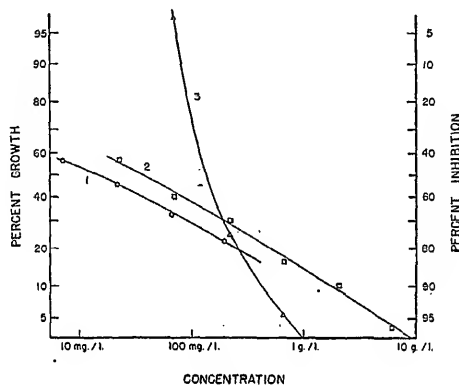


FIG. 2. Effects of hop resin constituents on *Sclerotinia fructicola*. 1. Humulon on mycelial growth. 2. Soft resin on mycelial growth. 3. Soft resin on spore germination.

It will be noted from Fig. 2 that the slopes of the dosage-response curves are very low. This means that while the concentration of resin giving 50% inhibition was low, a far higher concentration was needed to give 90% or 100% inhibition. It has been observed that this is commonly the case with fungi, but other antibiotics assayed against fungi in this laboratory have given somewhat steeper curves than these.

Because of the flatness of the dosage-response curve, the assay method lacks precision, for a slight vertical displacement of the curve makes a great change in the concentration corresponding to 50% growth. As a probable result, variations up to 2- or 3-fold have been encountered in different assays of the same material. The use of a standard fungicide (ethyl mercury phosphate) for comparison was of no value, since it gave a very much steeper dosage-response curve and was very uniform in its effect on the test fungi.

The fungi used as test organisms (Table I) were chosen to represent a number of genera and families. All (except perhaps *Aspergillus niger*) are pathogenic to crop plants. Slow-growing fungi were not used.

Bioassay Using Spore Germination

As discussed by Horsfall (13), the choice of organisms for spore germination assays is very limited. Of several which were tried, only *Sclerotinia fructicola* produced spores from which satisfactory counts could be made.

To obtain a spore suspension, 5-day old plate cultures of *S. fructicola* (on potato dextrose agar) were washed with 10 ml. of the following medium: 10% potato infusion, 0.1% KH_2PO_4 , adjusted to pH 5.8. The resulting suspension was shaken in a sterile tube to break up spore clumps. For each test, 0.5 ml. of spore suspension was placed in a test tube, to which was added 0.5 ml. of a given concentration of the preparation to be assayed. Tubes were made in duplicate. They were then corked and incubated for 3 hr. at 28°C. with gentle shaking. At the end of this time, 0.1 ml. of 1% HgCl_2 was added to each tube to prevent further germination. The tubes were then shaken vigorously to disperse spore clumps. From each tube a drop of medium was placed on a microscope slide and the proportion of germinated spores was determined by Horsfall's method (13). The concentration which would have suppressed germination of 50% of the spores was determined graphically and taken as a measure of the potency of the preparation. (Fig. 2, Curve 3).

Bioassay Against Yeasts

A few yeasts were streaked on plates containing various concentrations of soft resin. The medium and the preparation of the soft resin were the same as in the assays against mycelial growth of fungi. Absence of growth was the criterion of antibiotic activity.

RESULTS

Each fraction prepared from hop resin was assayed against mycelial growth of *Sclerotinia fructicola*. The activity (as mg./l. required to give 50% inhibition) is given in Fig. 1 for each fraction.

Soft resin was assayed against mycelial growth of 12 test fungi, and against germination of spores of one of these fungi. The results (Table I) show great variation in its activity against different fungi. Spore germination for *S. fructicola* was completely inhibited by a concentration which did not completely inhibit mycelial growth. (Compare Curves 2 and 3, Fig. 2.)

The final preparations were assayed against 4 of the test fungi (Table II). The concentrations used were not high enough to give complete inhibition. In a preliminary experiment, however, a mixture of humulon and "yellow resin" was tested at high concentrations. It had about the same effect at 2 g./l. as the unfractionated soft resin did at 18 g./l.

In general, the yeasts were much less sensitive than the other fungi to soft resin and its constituents. Of 7 yeasts, 5⁶ were not affected by any of the materials tested except in high concentrations (above 2 g./l.).

⁶ *Candida Krusei*, *Zygosaccharomyces mandschuricus*, *Debaryomyces membranacefaciens*, *Hanseniaspora melligeri*, *Hansenula anomala*.

TABLE I
Activity of Soft Resin Against Various Fungi

Organism	Concentration causing 50% inhibition ^a mg./l.	Inhibition by 18 g./l. ^a per cent
<i>Rhizopus nigricans</i>	40	99
<i>Pythium</i> sp.	500	99
<i>Rhizoctonia solani</i>	150	85
<i>Sclerotium bataticola</i>	80	90
<i>Aspergillus niger</i>	1600	70
<i>Aspergillus oryzae</i>	200	75
<i>Phytophthora citrophthora</i>	600	90
<i>Fusarium lycopersici</i>	300	70
<i>Penicillium digitatum</i>	250	70
<i>Botrytis cinerea</i>	200	65
<i>Alternaria citri</i>	900	60
<i>Sclerotinia fructicola</i>	40	98
<i>Sclerotinia fructicola</i> (spore germination)	150	100 ^b

^a Average of 2 expts. These data are based on visual inspection rather than measurement of colonies.

^b At 2 g./l.

Saccharomyces cerevisiae, however, was inhibited by "yellow resin" (2 g./l.) but not by lupulon, while *Torulopsis dattila* was inhibited by lupulon (2 g./l.) but only slightly by the same concentration of "yellow resin."

TABLE II
Activity of Hop Fractions Against Various Organisms

Test fungus	Average concentration producing 50% inhibition of test organism mg./l.						Inhibition by 2 g./l. (one assay) per cent
	Number of assays	Humulon	Lupulon	Yellow resin	Black resin	Hard resin, (unpurified)	
<i>Sclerotinia fructicola</i> ^a	4-5	20	20	50	>2000	>2000	100
<i>Sclerotium bataticola</i>	2	16	80	40	2000	2000	90
<i>Rhizopus nigricans</i>	2	9	140	40	1000	1000	97
<i>Aspergillus oryzae</i>	2	60	800	180	— ^b	— ^b	85

^a Data for *Sclerotinia fructicola* also appear in Fig. 1.

^b Negligible activity.

Humulon and "yellow resin" were analyzed for lead. The amount found was far too small to account for their antibiotic activity.

DISCUSSION

As previously explained, the "yellow resin" may have contained some humulon and lupulon. Data presented here do not show conclusively that it contained any other constituent having antifungal activity. It is known, however, that humulon and lupulon are not the only constituents of hop resin that are antibacterial (2).

The solubilities of humulon and lupulon at pH 5.9 are, respectively, 480 mg./l. and 12 mg./l. (14). Maximum inhibition of growth is, in most cases, given only by concentrations which are higher than these. At concentrations giving maximum inhibition, therefore, humulon and lupulon cannot exist entirely as solutes in the aqueous medium. Probably they are dispersed as colloids or adsorbed on solid particles in the medium.

Hop antibiotics are available in large quantities. Hops usually contain 5-10% humulon and 5-15% β -soft resin (2,15). The latter contains lupulon and the "yellow resin" described here.⁷ Our yield of lupulon (0.8% of the dry hops) is similar to that obtained by Walker (16) (0.8-1.0%), who isolated lupulon by extraction with dilute aqueous NaOH.

Since humulon, lupulon and the "yellow resin" are all soluble in 70% methanol, they could be separated in one operation from the inactive and highly colored "black residue." Such a preparation would have a high potency and would lack the dark color of the soft resin.

Hop antibiotics have been assayed against bacteria by Walker and Parker (9). Acid production by *Lactobacillus bulgaricus* was reduced 50% by 0.78 mg./l. of humulon, 0.21 mg./l. of lupulon, and 0.55 mg./l. of β -soft resin. Like the fungi, bacteria are not sensitive to hard resin (1). In contrast to the fungi, inhibition of bacteria increased very rapidly with increasing concentration of antibiotic (9). Additional experiments on the activity of these antibiotics against bacteria are in progress.

⁷ In hop analysis, the humulon is precipitated as its lead salt and is frequently reported as α -soft resin. The remainder of the soft resin, containing the lupulon, is reported as β -soft resin (1,2).

Certain advantages of hop antibiotics can be pointed out. Their use in beer suggests absence of oral toxicity. Their low solubility in water may make them desirable for surface applications where a more soluble substance would dissolve too rapidly. Lupulon is colorless, odorless, and tasteless. These properties suggest that it could be used medicinally, on foods, or on plants destined for use as food.

ACKNOWLEDGMENTS

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SUMMARY

A new method for the preparation of lupulon from soft resin of mature hops is described. It depends on spontaneous crystallization out of soft resin at low temperature, and purification by repeated recrystallization from hot 70% methanol.

All of the antifungal activity was found in the soft resin, from which it could be separated by extraction with hot 70% methanol.

Soft resin showed antibiotic activity against all of 12 species of fungi. The concentrations causing 50% inhibition of growth ranged from 40 to 1600 mg./l. Lupulon, humulon, and a yellow resinous fraction were active against the 4 fungi tested. Humulon was the most active, giving 50% inhibition at concentrations ranging from 9 to 60 mg./l. Under the test conditions complete inhibition of mycelial growth was frequently not found even at relatively high concentrations. Germination of spores of *Sclerotinia fructicola* was completely inhibited, however. Slight activity was found against 2 out of 7 yeasts.

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The Lipides of the Rat Brain and Liver in Choline Deficiency¹

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INTRODUCTION

Young rats growing on a choline-deficient diet develop a hemorrhagic syndrome which includes, among other symptoms, hemorrhages in the kidney, in various parts of the eye (1,2), in the cerebellum, and in the cerebral cortex (3). In view of the fact that the hemorrhages in the kidney are generally attributed to an inadequate supply of phospholipides due to faulty production in the liver (4), it was thought of interest to investigate the possibility that the hemorrhages in the central nervous system might be due to a similar cause.

The lipides of the brain are not affected to a great extent by the lipide composition of the diet (5,6). Some changes, however, have been obtained by cholesterol feeding (7) and in vitamin E deficiency (8). The phospholipide turnover in the brain is slower in the choline-deficient animal than in the animal receiving choline, but the difference is small in comparison to that observed in the liver and in the intestine (9,10). Choline does not increase the choline content of the brain when added to a normal diet (11). It appears, therefore, that the lipide composition of the brain is not influenced easily by dietary means. To our knowledge however, no data are available on the concentration of the various lipide fractions of the brain of animals fed a choline-deficient diet with or without choline supplements.

METHODS

Forty male hooded rats weighing 60-80 g. were paired and fed a diet composed of lard 20, Mazola 20, casein (Labco vitamin-free) 15, sucrose 20, starch 13, brewer's

¹ This work was made possible by a grant from The Upjohn Company, Kalamazoo, Michigan.

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yeast 5, salt mixture (Osborne and Mendel, 12) 4, and cod liver oil 3. A 0.2% supplement of choline hydrochloride was mixed with the diet of one rat in each pair. Eight male hooded rats weighing as much as the experimental animals and fed a Purina stock diet were used as additional controls. After 22 days of feeding the animals were killed by stunning and brains and livers analyzed according to Hack (13). Cholesterol was determined according to Bloor (14). Data on the lipide composition of the liver were desired as criteria of choline deficiency and as a help in the interpretation of the results of brain analyses.

RESULTS AND DISCUSSION

The type of diet had no significant influence on the weight of the animals and of the brains, although choline supplements produced the expected decrease in the weight of the liver of rats fed a choline-deficient diet (Table I).

TABLE I

Diet	Days	Food consumed	Rat weight	Brain		Liver	
				g.	per cent body	g.	per cent body
Purina	—	<i>Ad lib.</i>	101.4 (76-117)	1.5 (1.2-1.7)	1.5 (1.1-2.0)	6.1 (4.5-7.5)	6.0 (5.4-7.0)
Basal	22	116.5 (103.1-149.4)	102.4 (84-114)	1.7 (1.0-2.3)	1.6 (0.8-2.4)	6.1 (5.4-8.0)	6.0 (5.4-8.5)
Basal + 0.2% choline	22	116.4 (103.2-149.4)	104.6 (87.5-127.5)	1.8 (1.1-2.3)	1.7 (0.9-2.7)	5.3 (4.5-7.8)	5.1 (4.4-6.7)

The concentration of the brain lipides studied remained constant regardless of diet (Table II). This fact, together with the relative constancy of the brain lipide turnover, suggests that the hemorrhages observed by others in the central nervous system of choline-deficient rats are not due to inadequate supply of phospholipides. Hemorrhages of the brain, however, were not observed in these experiments.

The liver of rats fed the high-fat basal diet contained more total lipides and less total phospholipides, lecithin, and cholesterol than the livers of rats fed the Purina stock diet. Choline reduced the amount of total lipides, slightly increased the concentration of lecithin, but did not affect the concentration of cholesterol (Table III). That choline deficiency decreases the total phospholipides of the liver has been affirmed

TABLE II
Brain Lipides, g./100 g. Fresh Tissue

Diet	Total lipides	Total P lipides	Sphingo-myelin	Lecithin	Cephalin	Total Cholesterol
Purina	9.5 (8.0-12.5)	4.6 (3.7-5.5)	1.0 (0.9-1.1)	1.5 (1.1-1.8)	2.3 (1.9-2.7)	0.4 (0.2-0.6)
Basal	7.9 (6.2-9.6)	4.3 (3.5-5.3)	1.2 (0.7-1.5)	1.2 (0.7-1.6)	2.0 (1.5-2.4)	0.3 (0.2-0.4)
Basal + 0.2% choline	7.4 (5.7-9.7)	4.4 (3.2-5.6)	1.4 (0.7-1.6)	1.5 (0.9-1.8)	1.6 (1.0-1.8)	0.4 (0.3-0.5)

TABLE III
Liver Lipides, g./100 g. Fresh Tissue

Diet	Total lipides	Total P lipides	Sphingo-myelin	Lecithin	Cephalin	Total cholesterol
Purina	6.2 (5.4-6.9)	2.6 (2.2-2.7)	0.3 (0.2-0.4)	1.5 (1.2-2.0)	1.1 (0.8-1.4)	0.1 (0.06-0.2)
Basal	14.5 (10.2-16.1)	1.8 (1.3-2.0)	0.2 (0.1-0.3)	0.7 (0.5-1.2)	0.9 (0.7-1.3)	0.3 (0.25-0.35)
Basal + 0.2% choline	6.0 (4.4-6.6)	1.9 (1.3-2.2)	0.2 (0.1-0.3)	1.1 (0.7-1.4)	0.7 (0.6-1.3)	0.1 (0.05-0.12)

by some investigators (4,15) and denied by others (16,17). This lack of agreement may be due to differences in the age of the animals used and in the feeding technic, both of which greatly affect the results (15).

It may be worth noting that the weight of the rats was not affected by choline deficiency when the food consumption was equalized by the paired-feeding technic (Table I).

CONCLUSIONS

1. Choline deficiency does not affect the total lipid, total phospholipide, sphingomyelin, lecithin, cephalin, and cholesterol content of the rat brain.

2. Although brain hemorrhages were not observed in these experiments, it is believed that the hemorrhages observed by others in the central nervous system of choline-deficient rats are probably not due to insufficient supply of phospholipides.

3. Choline deficiency decreases the lecithin content of the liver. This decrease is reversed by choline supplements.

4. The cholesterol, sphingomyelin, and cephalin contents of the liver were not influenced by the diets used.

5. When the food consumption is equalized, choline deficiency does not affect growth.

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Studies of Pantothenic Acid Analogues

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INTRODUCTION

Mitchell (1) first observed an effect on bacterial growth produced by hydroxypantothenic acid, which Knight (2) later suggested represented a type of inhibition in which an analogue was capable of blocking the metabolite from its active enzyme site.

Snell (3) and Kuhn (4) synthesized pantoyltaurine and found it capable of inhibiting the growth of *L. arabinosus* in the presence of small amounts of calcium pantothenate. The inhibition was reversible.

McIlwain (5, 6, 7) reported the activity of numerous compounds structurally related to pantothenic acid. Some were similar in action to pantoyltaurine, while others inhibited growth irreversibly. Madina-veitia *et al.* (8) found panthydrazide to be the most effective of a series of pantoic acid amides.

The discovery of the acetylase activity of pantothenic acid (9) stimulated interest in displacers as possible pharmacological agents. Furthermore, it is probable that marked improvement in inhibitor metabolite ratios in this series would lead to valuable antimalarials.

EXPERIMENTAL

The following new analogues of pantothenic acid were prepared and tested. Salicyloyl β -alanide (salicyluric acid and benzoyl β -alanide were also tested for comparison with this compound), mandelyl β -alanide, acetyl mandelyl β -alanide, sodium salt of acetyl mandelyl β -alanide, N'-diacetylpantoylsulfanilamide, and γ -hydroxy-*n*-butyryltaurine (the activity of this compound was also compared with γ -hydroxy-*n*-butyryl- β -alanine).

Salicyloyl β -Alanide

To a solution of 17.6 g. (0.2 mole) of β -alanine in 50 cc. of water was added slowly, with stirring and cooling, salicyloyl chloride (0.1 mole) and 25 cc. of 50% NaOH over

a period of 15 min. The stirring was continued for another 45 min. The solution was acidified and an oil obtained. The mother liquor was decanted, and the residue dissolved in NaOH solution and treated on a steam bath for 15 min. The solution was acidified and the precipitate which occurred was collected, dried, washed with ether, and recrystallized twice from water. There was obtained a product of white needles, m.p. 152–153°C.

Anal.: Nitrogen (theoretical) 6.70%; (found) 6.78%.

Salicyluric Acid

This compound was prepared in essentially the same way as salicyloyl- β -alanide, only substituting glycine instead of β -alanine according to the procedure of Quick (10) and Fischer (11). m.p. obtained 155–158°C.

Benzoyl β -Alanide

This compound was prepared in essentially the same way as salicyloyl β -alanide using benzoyl chloride and β -alanine (12). The hydrolysis of the acetyl was omitted. m.p. obtained 118°–120°C.

N-Diacetylpantoylsulfanilamide

To a solution of 20.2 g. (0.1 mole) of *p*-nitrobenzene sulfonamide in 50 ml. of anhydrous pyridine kept on a steam bath was added 0.1 mole diacetylpantoyl chloride (13) slowly with stirring for a period of 15 min. The stirring and heating was continued for 30 min. longer. The reaction mixture was concentrated *in vacuo*, taken up in ethyl acetate, the ethyl acetate solution washed with dilute HCl and water, and then concentrated *in vacuo*. The oil was dissolved in ethyl alcohol and reduced catalytically, concentrated, and recrystallized from 50% acetic acid; brown needles, m.p. 180–185°C.

Anal: Sulfur (theoretical) 8.27%; (found) 8.45%.

Acetyl Mandelyl β -Alanide

To a cold solution of β -alanine containing excess barium hydroxide, acetyl mandelyl chloride was added slowly with stirring. After stirring for 2 hr., the reaction mixture was extracted with ethyl acetate and washed. The extract was concentrated *in vacuo*. A colorless oil was obtained.

Anal: N (theoretical) 5.28%; (found) 4.7%.

Mandelyl β -Alanide

Acetyl mandelyl β -alanide was hydrolyzed with dilute alkali. An oil was obtained.

Anal: N (theoretical) 6.28%; (found) 5.79%.

γ -Hydroxy-N-Butyryltaurine Barium Salt

A mixture of 9.6 g. of barium taurinate and 5 cc. of butyrolactone was heated in an oil bath at 150°C. for 5 hr. The residue was washed with hot CH₃OH and dried at 110°C. There was obtained a white crystalline powder.

Anal: Barium (theoretical) 24.62%; (found) 24.38%.

 γ -Hydroxy-N-Butyryl- β -Alanide Barium Salt

A mixture of barium β -alanide (0.1 mole) and 1.6 cc. of butyrolactone with 25 cc. of absolute CH₃OH was refluxed for 1 hr. according to the technique of Barnett and Robinson (14). The CH₃OH extract was concentrated to a small volume and precipitated with absolute ether. A white crystalline powder was obtained.

Anal: Barium (theoretical) 28.28%; (found) 28.15%.

All the bacteriological tests were carried out employing the technique described by Strong *et al.* (15). The activity of these agents was tested against *Lactobacillus casei* grown in the synthetic medium of Pennington *et al.* (16).

RESULTS AND DISCUSSION

*I. Compounds Having the Pantoyl Portion
of Pantothenic Acid Changed*

	Antibacterial index *
Mandelyl β -alanide	100
Acetyl mandelyl β -alanide	1000
Acetyl mandelyl β -alanide (Na salt)	1000
Salicyloyl β -alanide	100

* Ratio of inhibitor to metabolite producing 50% inhibition.

TABLE I
Anti metabolite Activity of Mandelyl β -Alanide

Calcium pantothenate γ /10 ml.	Mandelyl β -alanide (γ /10 ml)					
	0	1	10	100	1000	10,000
0	70	68	60	65	21	6
0.1	148	132	115	112	105	20
100.1	170	165	160	165	160	40
1000.1	180	170	170	167	165	58

Inoculum—0.1 ml. of a suspension in saline of a 24 hr. culture of *L. casei*.

Incubation—18 hr.; 37°C.

Previous work (17) showed that the inhibition of *E. coli* produced by mandelic acid, could be reversed by small amounts of pantothenic acid.

The activity was thought to be due to interference with a phase of bacterial metabolism involving pantothenate. By converting mandelyl radicals into the β -alanide, true displacement is possible.

Likewise, Ivanovics (18) showed that sodium salicylate and the methyl and phenyl esters of salicylate produced inhibition which could be reversed by pantothenic acid. By converting the salicyl radical into the β -alanide, true displacement is obtained.

II. Compounds Having the β -Alanine Portion of Pantothenic acid Changed

	Antibacterial index
1. γ -Hydroxy- <i>n</i> -Butyryltaurine	1000
2. N'-Diacetylpantoylsulfanilamide	100
3. Pantoyltaurine	1000

McIlwain (5, 6, 7) had observed that γ -hydroxy-*n*-butyryl- β -alanine was effective against organisms not requiring the preformed pantothenate molecule, whereas pantoyltaurine was ineffective against these organisms. His explanation of this difference was that pantoyltaurine was a true displacing agent, whereas γ -hydroxy-*n*-butyryl- β -alanine merely exhibited a non-competitive relationship with pantothenate.

With the results obtained with γ -hydroxy-*n*-butyryltaurine, the authors reached a different conclusion:

Some enzyme systems may utilize the pantoic portion of the molecule; therefore, an analogue with the pantoic acid portion changed might have specificity for an organism not requiring preformed pantothenic acid (*E. coli* for example). In other cases the β -alanine portion is of prime importance, and an analogue with the β -alanine changed, might be effective for the organism requiring preformed pantothenate, as in the case of *L. casei*. Therefore, if both portions were changed, the molecule should be effective for both types of organisms.

SUMMARY

1. A new series of pantothenic acid displacers were prepared and tested.

2. Salicyloyl- β -alanide, mandelyl- β -alanide, acetyl mandelyl- β -alanide (and the sodium salt), γ -hydroxy-*n*-butyryltaurine, and N'-diacetylpantoylsulfanilamide, all show marked antimetabolite activity.

3. Salicyluric acid, benzoyl- β -alanide, and Na salt of pantoic acid were inactive.

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The Inhibition of Enzyme Formation and Nitrogen Assimilation by Arsenate

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INTRODUCTION

The compounds hitherto best known for their ability specifically to inhibit synthetic and assimilative processes are sodium azide and the nitrophenols. Their effects upon oxidative and fermentative assimilation were studied by Clifton and his co-workers (1, 2, 3), by Winzler (4, 5), by Doudoroff (6), and by Foster (7, 8). The inhibition of nitrogen assimilation by azide was investigated by Winzler (9). Extensive studies by Krah1 and Clowes (10) demonstrated inhibition of embryonic development by nitrophenols and nitrocresols.

Inhibition of the formation of adaptive enzymes in yeast by azide and 2,4-dinitrophenol was shown by Reiner (11) and by Spiegelman (12). These workers also observed (13) that azide was able to stabilize the adaptive enzyme for galactose fermentation once it was formed.

The findings that azide prevents uptake of inorganic phosphate by yeast (14), and that it confers a limited protection against the poisoning of glucose fermentation by moniodoacetic acid (15), led to the suggestions that "the capacity of NaN_3 to prevent cellular utilization of metabolic energy for synthetic purposes resides in its ability to dissociate carbohydrate metabolism from the generation of energy-rich phosphate bonds," and that "azide disassociation of phosphorylation from carbohydrate fermentation occurs at the step catalyzed by glyceraldehyde oxidase (*sic*)."

It would be helpful in evaluating this theory if one could dissociate carbohydrate utilization from the generation of high-energy phosphate compounds by an independent method which is known to achieve such a result. Such a method is at hand in the use of arsenate, which was studied by Needham and Pillai (16) among others, and whose mechanism of action was elucidated by Meyerhof (17) and Warburg (18) and their co-workers.

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In this paper the effect of arsenate upon the formation and stability of an adaptive enzyme and upon the assimilation of nitrogen is studied, and its properties in this regard are compared with those of azide. The results strongly suggest that azide has another locus of action besides the one proposed in the theory quoted above.

EXPERIMENTAL METHODS

Manometric experiments were performed with the conventional Warburg equipment. Most experiments were performed under aerobic conditions, and the excess of CO_2 liberated over O_2 consumed was taken as a measure of the rate of aerobic fermentation. Anaerobic experiments were carried out under an atmosphere of N_2 gas.

Phosphate determinations were performed according to Lohmann and Jendrassik (19). Nitrogen determinations were made by the method of Levy and Palmer (20), using permutit according to Folin (21) to avoid interference with the oxidation by substances present in the samples.

Arsenate solutions were made by dissolving $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$ in distilled water and bringing the pH to 4.5 with HCl. Phosphate, when added, was in the form of KH_2PO_4 (pH 4.5). Adenosine triphosphate (ATP) was obtained about 90% pure from rabbit muscle by the method of LePage (22); the barium salt was converted to the potassium salt by the addition of K_2SO_4 in slight excess and removal of BaSO_4 by centrifugation.

The types of yeast studied were a strain of *Saccharomyces cerevisiae* (baking type, strain Kl) and one of *Saccharomyces carlsbergensis* (strain ClR). They were grown in yeast extract-peptone broth as previously described (23). Twenty-four or 48-hour cultures were harvested on the centrifuge, and freed from adherent medium by washing twice with portions of the liquid in which they were to be suspended (water or phosphate).

RESULTS

Inhibition of Adaptation

Arsenate prevents the formation of the adaptive enzyme for galactose fermentation which normally occurs on exposure of the yeast to galactose. This was the case in concentrations of arsenate from 10^{-2} *M*, the highest level tested, down to 2×10^{-4} *M*. As would also be expected from the mechanism of the arsenate effect (18), this inhibition could be reversed by addition of inorganic phosphate, the degree of reversal depending on the arsenate concentration at a given phosphate level³. No reversal was observed when the same amount of phosphate was added at a later time, when the controls were beginning to adapt. Some typical results are shown in Table I.

³ Previous work (unpublished) by Mr. Sussman has established the inhibition by arsenate of carbohydrate assimilation and that this inhibition can be reversed by inorganic phosphate.

TABLE I
*Inhibition of Aerobic Galactose Adaptation by Arsenate
 and Reversal by Phosphate*

Time in minutes	Control	Arsenate 10^{-3} M			Arsenate 10^{-2} M		
		No phosphate	Phosphate	Phosphate added at 180 mins.	No phosphate	Phosphate	Phosphate added at 180 mins.
150	16	0	12	0	0	0	0
220	29	2	39	2	0	9	0

Figures are $Q_F^{O_2}$ (μ l. excess CO_2 /hr./mg. dry weight of yeast). Time is in mins. of incubation. Yeast 2 mg. dry weight. Galactose 3%. Phosphate (when indicated) 0.05 M KH_2PO_4 . Total volume, 2 ml. Gas phase, air.

It will be noted that the addition of phosphate sometimes raises the activity in the arsenate-poisoned system above that of the control. This is probably related to the fact, which we have frequently observed, that adaptation is slower and less regular in the absence of phosphate. That this is not due simply to the buffering effect of the phosphate was shown by a comparison of rates in phosphate and in succinic acid-sodium succinate buffer at the same pH. The data are summarized in Table II.

TABLE II
Effect of Phosphate on Rate of Galactose Adaptation

Time	Phosphate	Succinate
145	15	2
205	26	6

Significance of figures and conditions as in experiment of Table I. Succinate: Succinic acid-sodium succinate buffer, pH 4.5, 0.05 M.

Inhibition of Nitrogen Assimilation

Yeast cells metabolizing carbohydrate are able to assimilate ammonia nitrogen from the suspension medium, with the eventual formation of amino acids, proteins, and other nitrogenous compounds [see, for example, (24)]. The effect of arsenate on this assimilation was studied, using a limiting amount of nitrogen to avoid complication of the experiment by extensive cell multiplication. Glucose served as the source of carbohydrate; the incubation was carried out in tubes at 30°C. Since domestic strains of yeast require compounds of the "bios" type for

nitrogen assimilation, a small amount of yeast extract was added (Yeast Extract No. 3, Anheuser-Busch). At the end of the incubation period, the cells were removed by centrifugation in the cold, and aliquots of the supernatant medium were analyzed for ammonia nitrogen, after separation from residual glucose and other interfering substances as described under "Experimental Methods."

The data presented in Table III indicate that arsenate inhibits nitrogen assimilation just as it does enzyme formation, and that this inhibition, like the other, can be reversed by inorganic phosphate.

TABLE III
*Inhibition of Nitrogen Assimilation by Arsenate
and its Reversal by Phosphate*

Sample	Nitrogen	Decrease in nitrogen	Decrease in nitrogen
	γ	γ	per cent
Zero time	149	—	—
Control, phosphate	6	143	97
Control	53	96	65
Arsenate $2 \times 10^{-4} M$, phosphate	0	149	100
Arsenate $2 \times 10^{-4} M$	134	15	10
Arsenate, $10^{-3} M$, phosphate	0	149	100
Arsenate $10^{-3} M$	144	5	3

Figures refer to ammonia nitrogen in suspension medium. Yeast, 2 mg. dry weight. Nitrogen added as $(\text{NH}_4)_2\text{SO}_4$. Phosphate (when indicated) 100 γ as KH_2PO_4 . Glucose 40 mg. Yeast extract, diluted 1:50, 0.05 ml. Time of incubation 3 hrs. 25 mins. Temperature 30°C.

Effect of Arsenate on Enzyme Stability

One of the most interesting and obscure properties of azide is its ability not only to prevent enzyme formation but also to prevent enzyme breakdown. It has been suggested (13) that the two effects have the same mechanism—that breakdown of one enzyme results from the competitive formation of another, so that depriving the cell of the energy needed to form enzymes also, in effect, deprives it of the ability to break down enzymes already present. The effect of arsenate on enzyme stability would seem a good test for this hypothesis.

Yeast cells grown in galactose instead of glucose, and hence possessing their full complement of adaptive enzyme, were used in these experiments. The suspension of

washed cells was incubated in Erlenmeyer flasks at 30°C. in the presence of a small amount of glucose, with and without added arsenate. Samples were taken at zero time and after a number of hours, and tested for the ability to ferment galactose, after being washed 3 times with $M/15$ KH_2PO_4 and resuspended in the phosphate buffer.

Table IV summarizes the data from several typical experiments. In every case, the effect of arsenate was to accelerate the breakdown of the galactose enzyme which is induced by the removal of galactose. The data of Expt. 3 show that phosphate, which has no effect on the break-

TABLE IV
Effect of Arsenate on the Stability of Adaptive Galactozymase

	Time	Control	Arsenate 2 × 10 ⁻⁴ M		
Experiment 1	hrs				
	0	188	—		
Experiment 2	4	122	63		
	0	268	—		
	4	79	37		
	Phosphate		No phosphate		
	Time	Control	Arsenate 10 ⁻² M	Control	Arsenate 10 ⁻² M
Experiment 3	hrs				
	0	294	—	—	—
	4	150	88	150	31

Yeast (*S. carlsbergensis*, grown in 8% galactose broth) incubated in flasks for periods indicated by "Time." Glucose 0.25%. Phosphate (when indicated) 0.067 M as KH_2PO_4 . Arsenate in concentrations indicated. Samples washed and suspended in KH_2PO_4 , tested for rate of anaerobic fermentation in 3% galactose. Figures are $Q_{\text{CO}_2}^{\text{N}_2}$ ($\mu\text{l. of CO}_2/\text{hr./mg. yeast}$).

down in the absence of arsenate, has considerable ability to counteract the effect of arsenate.

The possibility that the arsenate might simply be acting as an inhibitor of fermentation, although unlikely, was considered. Accordingly, the effect of comparable arsenate concentrations on the fermentation rate of fresh yeast on glucose and galactose was tested. No effect was found in the presence of phosphate, as was to be expected; in the absence of phosphate, the maximal inhibitory effects found ranged from 10 to 15%. Since the tests of incubated cells were carried out in phos-

phate buffer, and since the arsenate effects found are much larger than 10 or 15%, they apparently cannot be accounted for on this basis.

The results are what one would expect if energy is required for the maintenance as well as the formation of enzymes. If this is true, arsenate should be able to accelerate the breakdown of the glucose system, which is normally much more stable than the adaptive galactose system. This possibility was tested in the manner just outlined, using glucose-grown cells and testing samples for anaerobic glucose fermentation rate. Incubations were also carried out in the presence of small amounts of galactose, since the glucose system is known to break down during adaptation to galactose (25). The data given in Table V are in

TABLE V
Effect of Arsenate on Stability of Glucose-Fermenting Enzymes

	Time	No galactose		Galactose 0.25%	
		Control	Arsenate 10 ⁻² M	Control	Arsenate 10 ⁻² M
Experiment 1	hrs. 0	362	—	—	—
	5.5	256	168	216	108
Experiment 2	0	237	—	—	—
	5	211	159	200	132

Conditions of incubation similar to experiments of Table IV, except for omission of glucose. Galactose and arsenate concentrations as indicated. Glucose-grown yeast, *S. cerevisiae* in Expt. 1 and *S. carlsbergensis* in Exp. 2. Samples tested for anaerobic fermentation rate in 3% glucose. Figures are $Q_{CO_2}^{N_2}$ (defined as in Table IV).

agreement with expectation, showing the ability of arsenate to induce glucozymase breakdown and to accelerate the smaller effect of galactose.

Arsenate and Intracellular Phosphate

Since arsenate presumably competes with the intracellular phosphate it seemed of interest to determine whether this phosphate could be retained by the arsenate-poisoned cell. Accordingly, at the conclusion of the experiments of Table IV, the supernatant medium which was removed from the cells before they were tested was used for phosphate and arsenate determinations. The samples with and without arsenate were treated according to the usual methods for a phosphate determin-

ation, and at the same time a portion of arsenate solution equivalent to the arsenate in the experimental samples was treated in the same way. In our version of the procedure, we use Photol (Merck) as the reducing agent, and develop color in the samples for 60 mins. at room temperature before reading. This seems to eliminate the difficulties sometimes encountered in the simultaneous determination of phosphate and arse-

TABLE VI
Loss of Inorganic Phosphate by Yeast in Presence of Arsenate

	Sample	Phosphate determined γ
Experiment 1	1—Control	1
	2—Arsenate $2 \times 10^{-3} M$	9
	3—Equivalent arsenate (4 ml. 2×10^{-3} arsenate)	2
	Phosphate lost (2-1-3)	6
Experiment 2	1—Control	0
	2—Arsenate $10^{-2} M$	18
	3—Equivalent arsenate (4 ml. $10^{-2} M$ arsenate)	8
	Phosphate lost (2-1-3)	10
	Contents of Sample	
Experiment 3 (recovery of arsenate and phosphate)	1 γ P	1
	1 γ P and 4 ml. $2 \times 10^{-3} M$ arsenate	3
	1 γ P and 4 ml. $2 \times 10^{-2} M$ arsenate	9
	4 ml. $2 \times 10^{-3} M$ arsenate	2
	4 ml. $10^{-2} M$ arsenate	9

Expts. 1 and 2: Phosphate determinations carried out on the suspension medium in which yeast (16 mg. dry weight) had been incubated for 4 hrs. alone or with the arsenate concentration indicated, together with 0.25% glucose. Sample 3 in each case consists of an arsenate solution containing the same amount of arsenate that had been added to Sample 2.

nate. This is indicated by the additivity which was found in experiments performed in order to be certain that our results were not due to such a difficulty.

Table VI shows that an appreciable amount of phosphate leaks from the cells during 4 hrs. exposure to arsenate. The value of 0.4–0.6 γ P/mg. dry weight of yeast compares with the average value of about 5 γ /mg. for the total acid-soluble phosphate of the yeast. The loss probably comprises most of the yeast inorganic phosphate.

Antagonism of Adenosine Triphosphate to Arsenate

If the action of arsenate in preventing phosphate esterification accounts for its ability to inhibit enzyme formation, the addition of a sufficient amount of adenosine triphosphate as an independent energy source for the cells should circumvent this inhibition. The data of Table VII show that this is the case, and, in addition, the stimulation of

TABLE VII

Reversal of Arsenate Inhibition of Galactose Adaptation by Adenosine Triphosphate

	Expt. 1
Incubation time, mins.	200
Control	19
Arsenate 10^{-3} M	0
Arsenate 10^{-3} M, ATP 1.4×10^{-2} M	23
ATP 1.4×10^{-2} M	39
	Expt. 2
Incubation time, mins.	300
Control	52
Arsenate 10^{-2} M	0
Arsenate 10^{-2} M, ATP 1.4×10^{-2} M	110
Arsenate 10^{-2} M, phosphate 5×10^{-2} M	74
Arsenate 10^{-2} M, phosphate 100 γ	0
ATP 1.4×10^{-2} M	212

Conditions similar to those of Table I.

Expt. 1: Yeast, *S. cerevisiae*. Figures are $Q_F^{O_2}$ (μ l. excess CO_2 /hr./mg. yeast).

Expt. 2: Yeast, *S. carlsbergensis*. Vessels flushed with N_2 after 300 mins. of aerobic incubation. Figures are $Q_{CO_2}^{N_2}$ (μ l. CO_2 /hr./mg. yeast).

adaptation by the unpoisoned cells, previously reported by us (11), is again exhibited, as well as the phosphate effect first shown in Table I. Since the ATP used contained as impurity a small amount of inorganic phosphate, equivalent to about 90 γ P in the quantity of ATP used per vessel, a control containing 100 γ P was included in the second experiment. This amount of P had no effect against the arsenate, and so cannot account for the effect of ATP itself. Moreover, even 0.05 M phosphate was a less effective antagonist of arsenate than was ATP.

If the mechanism of azide inhibition of adaptation, like that of arsenate, depended on its ability to prevent phosphate esterification, adenosine triphosphate should also reverse the azide effect. Such a reversal was carefully sought, but was not found to a measurable extent

in any experiment, or under any set of conditions tried. The aerobic experiments being open to the objection that azide also inhibits oxidative processes, they were checked by completely anaerobic experiments, which were equally negative. Azide was found to have no effect on the spontaneous breakdown of ATP in experiments performed to check this point. Since azide apparently inhibits the adenosine triphosphatase of yeast to some degree (26), it is unlikely that the azide could have made the ATP unavailable for the cells by promoting its breakdown.

Arsenate and Maltose Adaptation

Another well-known adaptive enzyme is concerned with the fermentation of maltose (27). Because of its greater instability as compared to galactozymase, it was not studied extensively. Data from an experiment designed to show that the same general phenomena occur with

TABLE VIII
Inhibition of Maltose Adaptation by Arsenate

Incubation time, mins.	180
Control	258
Arsenate 10^{-2} M	0
Arsenate 10^{-2} M, phosphate 5×10^{-2} M	18
Arsenate 10^{-2} M, ATP 1.4×10^{-2} M	15

Conditions as in experiments of Table VII, but with 3% maltose instead of 3% galactose. Yeast, *S. cerevisiae*. Vessels flushed with N_2 after 180 mins. incubation. Figures are $Q_{CO_2}^{N_2}$.

this system are summarized in Table VIII. The very small power of phosphate or ATP to restore activity to the arsenate-poisoned system contrasts with the data of Table VII.

DISCUSSION

Arsenate and azide have in common their inhibition of certain synthetic processes. They differ sharply in two important respects. Arsenate, but not azide, can be counteracted by adenosine triphosphate and by inorganic phosphate. On the other hand, arsenate, unlike azide, does not stabilize enzymes in the absence of their substrates, but, on the contrary, accelerates their breakdown.

In the case of arsenate, a mechanism of action *in vitro* is known and widely accepted; this mechanism results in the dissociation of carbo-

hydrate metabolism from phosphate esterification. In the case of azide, no mechanism has been firmly established by *in vitro* methods; only certain indirect inferences have been drawn from data on intact cells.

It is possible, of course, that arsenate acts upon the intact cell in a manner essentially different from the mechanism in cell-free preparations. However, there seems at present no need to assume such a difference, particularly since all the effects observed were predicted from, and are consistent with, the properties of the cell-free model. In any case, the distinction between the modes of action of arsenate and azide is independent of this question; and the data make it quite unlikely that azide can prevent synthesis simply by preventing the accumulation of high-energy phosphate compounds.

These considerations do not rule out the possibility that azide may act at the level of the triose phosphate dehydrogenase and thereby inhibit phosphate esterification. But they result in a strong probability that azide has some other locus of action, and that this locus is involved in the ability of azide to inhibit enzyme synthesis and breakdown. No indications of its nature are as yet available.

The loss of phosphate from arsenate-poisoned cells, although an incidental observation, is of interest. Such leakage occurs to a lesser extent with azide, but not with such poisons as fluoride or moniodoacetic acid (28). This makes it unlikely that non-specific damage to the cell or the cell surface is responsible for the effect. It is well known that metabolic activity (and presumably the associated phosphate esterification) is required for the uptake of inorganic phosphate by yeast (29). Hence, it seems reasonable that utilization of intracellular phosphate should be necessary for its retention, although endogenous metabolism alone seems to ensure such utilization to a fairly adequate extent (30).

One might speculate whether the loss of phosphate would suffice to explain the effect of arsenate on synthesis. This would seem to be largely a question of terminology. If a cell is prevented from esterifying its phosphate, it cannot perform energy-requiring syntheses; if it also loses the unused phosphate, *a fortiori* it cannot esterify it. The equivalence of the two points of view is a logical consequence of the fact that arsenate inhibition is competitive.

SUMMARY

Arsenate is shown to inhibit the formation of adaptive enzymes and the assimilation of ammonia nitrogen by yeast cells. Unlike azide, an-

other inhibitor of synthesis, it does not stabilize adaptive enzymes in the absence of their substrate, but accelerates their breakdown and that of "constitutive" enzymes as well. Again unlike azide, its inhibitory action is not only reversed by inorganic phosphate, but also by adenosine triphosphate.

The implications of these findings for the mechanism of azide action are briefly discussed.

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The Urethan Inhibition of Invertase Activity in Relation to Hydrostatic Pressure¹

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INTRODUCTION

The rate of sucrose hydrolysis by yeast invertase has been studied in relation to pressure, temperature, and pH (1). Pressures up to 680 atm. have a marked effect only under conditions of temperature or pH which cause a partial diminution in activity of the enzyme, *e.g.*, at 35° or 40°C. and pH 7.03, where the application of pressure increases the reaction velocity by reversing an equilibrium change that takes place with a volume increase of reaction of about 69 cc./mole, from active to inactive states of the catalyst.

The present study is with particular reference to the influence of pressure on the urethan inhibition at both pH 4.5 (optimal) and pH 7.0, as well as at different temperatures. Urethan and related substances apparently catalyze the reversible and (or) irreversible denaturations of intracellular enzyme systems and certain extracted proteins through reactions opposable by hydrostatic pressures of 680 atm. and less (2-5).

METHODS

As in the previous study (1), the enzyme preparation consisted of "Difco" yeast invertase solution, usually diluted to 0.2%, in the reaction mixture containing 10% sucrose. At low temperatures, or at alkaline pH, somewhat larger concentrations of enzyme were used, and the observed rates were multiplied by the appropriate factor for comparison with the rates obtained with the lower enzyme concentration. In the range of concentrations employed, 0.2-1.0% solution of the commercial preparation, the velocity of the reaction is proportional to the amount of enzyme, under both normal and increased pressure (1). Solutions were buffered by acetate at pH 4.5 or phos-

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phate at pH 7.0, in a final concentration of 0.02 *M*. With different concentrations of acetate between 0.01 and 0.10 *M*, at 42°C. and pH 4.5, the rates of the reaction at either normal or increased pressure respectively, were found to be independent of the buffer concentration. With phosphate, however, at 35°C. and pH 7.0, the rate varies with buffer concentration, reaching at 0.02 *M* a maximum that is some 20% faster than it is at 0.01 or 0.32 *M*. The quantitative influence of pressure under these conditions also varies with the phosphate concentration, in general decreasing with increase in concentration, although the difference is small between 0.01 and 0.04 *M*.

RESULTS AND DISCUSSION

At the optimum pH of 4.5, the rate of hydrolysis is typically first order, following a short "lag period," throughout the range of temperatures from below room temperature to about 60°C., where the activity

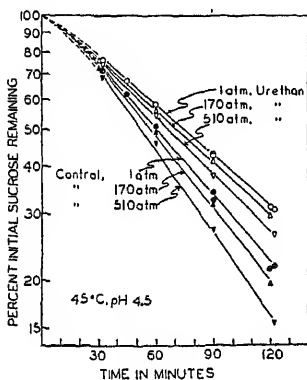


FIG. 1.

Fig. 1. Rate of hydrolysis of 10% sucrose by 0.2% Difco invertase solution, and the influence of 0.5 *M* urethan, under normal and increased hydrostatic pressure, at pH 4.5 and 45°C. The curves at normal pressure show points from two repeated experiments.

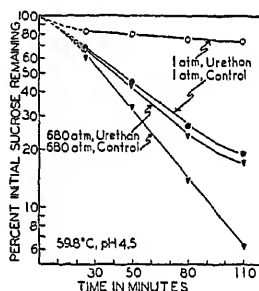


FIG. 2.

Fig. 2. Rate of hydrolysis of 10% sucrose by 0.2% Difco invertase solution, and the influence of 0.5 *M* urethan, under normal and 680 atm. hydrostatic pressure, at pH 4.5 and 59.8°C.

of the enzyme at normal pressure reaches a maximum. At temperatures above 60°C. the rate tends to decrease as the reaction proceeds, and this decrease in rate with time is especially marked in the presence of 0.5 *M* urethan. At lower temperatures, however, the rate of hydrolysis remains logarithmic in the presence of the same concentration of urethan. Representative data are shown in Figs. 1 and 2, which also illustrate

the influence of increased hydrostatic pressure on the course of the reaction. Under pressure, the reaction is accelerated both with and without urethan. Although the acceleration caused by a given increase in pressure is approximately the same in the control and urethan-containing solutions at moderate temperatures, such as 45°C., it is evident that, at temperatures near the normal optimum of 60°C., urethan causes a much greater inhibition and one which is more strongly opposed by pressure.

At pH 7.0 the activity of the enzyme is reduced in comparison with that at pH 4.5, and reaches a maximum under normal pressure at about

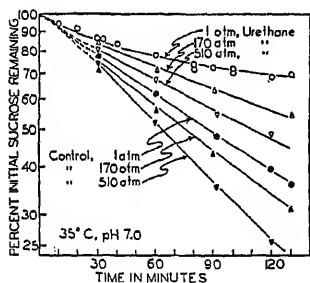


FIG. 3.

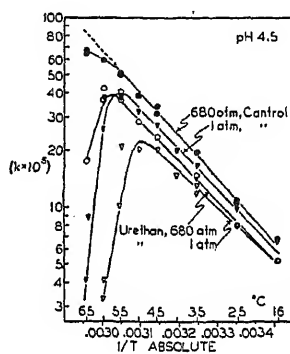


FIG. 4.

FIG. 3. Rate of hydrolysis of 10% sucrose by 1.0% Difco invertase solution, and the influence of 0.5 *M* urethan, under normal and increased hydrostatic pressure, at pH 7.0 and 35°C. Points from repeated experiments are shown on the uppermost curve.

FIG. 4. The rate of sucrose hydrolysis by invertase during the logarithmic period as a function of temperature under normal and 680 atm. pressure, with and without 0.5 *M* urethan, at pH 4.5. The first order rate constants in reciprocal seconds on the logarithmic scale of the ordinate are plotted against the reciprocal of the absolute temperature on the abscissa. No detectable hydrolysis occurred in the absence of the enzyme.

35°C., rather than 60°C. The reduction in activity at the more alkaline pH may be partially counteracted by hydrostatic pressure, as noted previously (1), and the influence of pressure becomes especially marked with rise in temperature above 35°C. The time course of the reaction follows a logarithmic rate at 35°C., but the addition of 0.5 *M* urethan results in a decreasing reaction rate as the hydrolysis proceeds. Pressure

opposes this decrease in velocity with time, and accelerates the hydrolysis both with and without urethan. Representative data are illustrated in Fig. 3.

With respect to the cause for the decrease in velocity constant during the course of the reaction in the presence of urethan, two interpretations are possible: (1), the drug promotes a progressive destruction of the enzyme, or (2), the combination between the drug and its site of inhibitory action occurs slowly, so that it continues to take place as the hydrolysis proceeds. The former appears the more likely explanation, however, since it has been shown that urethan acts almost immediately in causing the reversible inhibition of intracellular luminescence, and accelerates uniformly the thermal destruction of the bacterial luminescent system (5), as well as of tobacco mosaic virus protein.² Moreover, it is only under conditions of temperature and pH approaching those where a rate process of destruction of the enzyme occurs in absence of urethan that the drug causes a progressive decrease in reaction velocity. Under other conditions, the inhibition remains the same and the rate logarithmic for the period between about the first 15–80% or more completion of substrate hydrolysis. Finally, although it has not been shown previously that pressure will retard the accelerated thermal destruction of proteins in the presence of urethan, it has been found that pressure opposes the thermal denaturation of human serum globulin in presence of small concentrations of alcohol (2), whose action in important respects resembles the action of urethan (5). Recent observations on tobacco mosaic virus indicate that pressure retards the thermal denaturation that is accelerated by urethan in concentrations similar to those used in the present study.²

The influence of temperature, increased pressure, and 0.5 *M* urethan on the rate of hydrolysis is summarized in Fig. 4. The velocity constants were calculated from the logarithmic portion of the curve relating the per cent sucrose remaining against time, as shown in Figs. 1–3. This period usually extended from about the first 20 to at least 120 min. of reaction.

In those cases where the rate progressively decreased, the observed rate was obviously limited by more than one reaction, and it was, therefore, impossible to compute readily the desired constant. Consequently, the points on the curve beyond the temperature of maximum rate in each case are subject to large error. They were obtained from the slope of the best straight line drawn by inspection through the points

² To be published.

representing the per cent sucrose remaining during the period between 20 and 110 min. after the start of the reaction. In spite of the inaccuracy of some of the points, however, it is apparent that, first, urethan acts in a manner that results in a lowering of the temperature for maximum rate, while, second, pressure opposes this action and raises the temperature of maximum rate of hydrolysis both in the presence and absence of urethan. Furthermore, it is apparent that, throughout the lower temperatures, there is no large influence of either temperature or pressure on the urethan inhibition. Thus, it is evident that the inhibitory effects of urethan take place through more than one reaction.

The data are treated analytically in Table I, in accordance with the formulations for enzyme inhibition, volume change, *etc.*, derived earlier (3, 4, 5). The rate constants, k , have been computed in the manner described in the preceding paragraph. At 16 and 25°C., the effect of pressure is small and within an experimental error of less than 10%. At higher temperatures the rate constant in all cases is greater under 680 atm. than under normal pressure, and the difference increases with rise in temperature, this increase becoming very marked above 55°C. in the absence of urethan, or above 45°C. in presence of 0.5 M urethan. The calculations of volume change, therefore, would seem to indicate a volume decrease of activation, amounting to 10 cc. or less at the lower temperatures, and fairly rapidly increasing to the order of 100 cc./mole at the higher temperatures. The interpretation of the smaller effect is uncertain: it could mean either a direct acceleration of the catalytic activity of the enzyme, or the opposal of an inactivation equilibrium that possibly arises from impurities in the crude invertase preparation and involves a small volume increase of reaction in going to the inactive state. The large effect of pressure, on the other hand, is of the same magnitude as in protein denaturations referred to above, and it is most likely that this represents the opposal by pressure of the thermal denaturation of the enzyme that proceeds with a large volume increase. To what extent this denaturation is an equilibrium reaction, and to what extent a rate process of irreversible destruction cannot be distinguished clearly at present. For this reason, the volume changes listed as ΔV in Table I for the total effect at the high temperatures are of uncertain quantitative significance.

With particular reference to the Urethan inhibition at low temperatures, Table I shows that the inhibition is actually slightly greater under 680 atm. than under normal pressure. The volume change, heat and entropy of the reaction, however, are small, as indicated by the thermodynamic considerations in the following paragraph. This re-

TABLE I
Inhibition of Invertase Activity by 0.5 M Urethan in Relation to Temperature under Normal
and 680 Atmospheres' Hydrostatic Pressure

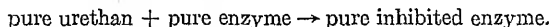
Temp. °C.	Rate constant, sec ⁻¹ × 10 ⁵				Urethan inhibition 1 atm. $\left(\frac{k_1}{k_2} - 1\right)$ = K_1 atm.	Urethan inhibition 680 atm. $\left(\frac{k_3}{k_4} - 1\right)$ = K_{680} atm.	ΔV , Control $\frac{RT}{\partial p} \ln \left(\frac{k_1}{k_2}\right)$ cc.	ΔV , with urethan $\frac{RT}{\partial p} \ln \left(\frac{k_2}{k_1}\right)$ cc.	Urethan inhibition at 680 atm. $\frac{\text{Urethan inhibition}}{K_{680} \text{ atm.}}$ = $\frac{K_1}{K_{680}}$	$\frac{\Delta V}{E} + \frac{\Delta U}{-EU}$
	k_1 1 atm.	k_2 1 atm. + urethan	k_3 680 atm.	k_4 680 atm. + urethan						
16	6.75	5.25	6.85	5.25	0.285	0.303	-0.5	0	1.06	-2.0
25	10.0	8.05	10.6	7.95	0.242	0.333	-2.1	+0.3	1.38	-11.6
25	10.0	8.15	11.0	8.45	0.228	0.301	-3.4	-1.2	1.32	-10.0
35	16.9	13.3	19.8	14.8	0.271	0.338	-5.8	-3.8	1.25	-8.2
45	27.4	20.2	34.9	24.2	0.356	0.441	-9.15	-7.05	1.24	-8.3
50	32.3	20.3	39.0	28.4	0.591	0.373	-7.35	-13.0	0.632	+18.0
55	38.6	21.6	50.8	37.1	0.788	0.370	-11.9	-21.3	0.469	+30.0
55	41.3	10.3	51.7	41.3	3.015	0.255	-8.5	-54.7	0.083	+98.0
60	38.6	4.2	60.0	43.1	8.195	0.391	-17.5	-93.5	0.048	+122.0
60	26.3	3.29	60.5	37.7	6.992	0.606	-33.4	-97.5	0.087	+98.0
65	12.3	0.18	67.5	18.0	57.4	2.74	-69.0	-187.	0.048	+124.0
65	4.17	0.147	65.9	17.6	27.4	2.74	-120.	-194.	0.100	+94.0

action apparently does not involve extensive changes in the enzyme protein, in contrast to the inactivation that takes place at the higher temperatures. The latter has been interpreted as a drastic alteration of molecular configuration through an unfolding of the protein in the process of denaturation (1).

Assuming that at temperatures below 50°C., pH 4.5, urethan (*U*) acts through an equilibrium (*K*) with the enzyme (*E*), then

$$K = \frac{[EU]}{[E][U]} = e^{-\Delta F/RT}.$$

The values of $K(U)$ are given in Cols. 6 and 7 of Table I. If concentrations are expressed as mole fractions and the solutions are ideal for all substances involved in the reaction, then ΔF is the free energy of reaction for the reactants and product in their pure states, *i.e.*, ΔF for the reaction:



In this way the extraneous effect of the entropy of mixing of the urethan with the solvent is eliminated. A 0.5 *M* urethan solution in water has a mole fraction of 0.0089, and, at this concentration, the inhibition at temperatures between 16 and 35°C. is approximately 0.25:

$$K(U) = \left(\frac{k_1}{k_2} - 1 \right) = \frac{[EU]}{[E]} \cong 0.25.$$

Thus, $K = 28$, and $\Delta F = -2000$ calories $= \Delta H - T\Delta S$.

The data indicate that, below 50°C., ΔH is positive, since the inhibition $(k_1/k_2 - 1)$ increases slightly with rise in temperature. The true value of ΔH cannot be found from these data, but it is probably of the order of $+2000 \pm 2000$ calories. This leads to a ΔS of $+13 \pm 7$ entropy units. If the ratio between the number of urethan molecules combining with the enzyme molecule is greater than unity, assumed above, then the figure of 13.7 ± 7 entropy units would have to be multiplied by the figure for the appropriate ratio. However, in a few experiments with reference to the relation between amount of urethan and of inhibition under given conditions, it appeared that the inhibition is closely proportional to the first power of the drug concentration, except at excessively high concentrations. Thus, the above value for the ent-entropy, although still an approximation, is as nearly correct as the data permit.

ACKNOWLEDGMENTS

The authors are pleased to acknowledge the assistance of Miss Virginia Miller with the experiments, and of Mrs. Sally Schlegel in the calculation of some of the data.

SUMMARY

At the optimum pH of 4.5, the inhibition of invertase activity by 0.5 *M* urethan is scarcely influenced by temperature from 16–45°C., but is slightly greater under 680 atm. than at normal pressure, indicating a volume change of about – 10 cc./mole in the reaction between urethan and enzyme. At this pH, the inhibition rapidly increases with rise in temperature between 50 and 65°C., probably involving both irreversible and reversible actions, but this increase in inhibition is opposed by 680 atm. hydrostatic pressure to an extent that would indicate a volume increase of reaction of about 100 cc./mole if the inactivation had resulted wholly from a mobile equilibrium. Pressure opposes by a similar magnitude the thermal reduction in activity by temperatures above 60°C. in absence of urethan.

At pH 7.0, the enzyme activity is less, and the urethan inhibition greater, than at corresponding temperatures, pH 4.5. Pressure causes a pronounced increase in enzyme activity, both with and without urethane.

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The Thermal Denaturation of Tobacco Mosaic Virus in Relation to Hydrostatic Pressure ¹

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INTRODUCTION

It has been shown (1) that tobacco mosaic virus (TMV) in neutral solution denatures as a first order rate process at temperatures of 67–73°C. With a given initial virus concentration of 6 mg./cc., the rate varies as a simple function of temperature, in accordance with an activation energy of about 153,000 cal. At 30°C., high pressure accelerates denaturation, also with the kinetics of a first order reaction, but the rate is not a simple function of pressure at this temperature. It goes through a maximum at about 7500 atm., and is relatively slow at either 5000 or 10,000 atm. (2).

Previous studies with other proteins and biological systems have shown that, while denaturation generally occurs under very high pressures at room temperature [*cf.* reviews by Macheboeuf and Basset (3), Cattell (4), and Bridgman (5)], the rate of thermal denaturation may be considerably retarded by pressures of 680 atm. and less (6). We have, therefore, undertaken a study of the relation of hydrostatic pressure to the rate of the thermal denaturation of TMV in an effort to reach a clearer understanding of the reactions involved under various conditions. This paper deals with the influence of pressures up to 680 atm. on the rate of TMV denaturation at 68.8 and $72.5 \pm 0.02^\circ\text{C.}$ in neutral solution.

METHODS

The data reported herein were all obtained with a recently prepared, ultracentrifugally isolated virus solution ² stored at 1–3°C. for a maximum of a month in distilled

¹ Aided in part by a grant from the American Cancer Society, through the Committee on Growth of the National Research Council.

² The authors are greatly indebted to Dr. W. M. Stanley and his associates for their cooperation and assistance in obtaining the purified virus preparation.

water. A stock solution containing 12 mg. protein nitrogen/cc. was centrifuged for 10 min. at 12,700 *g* to remove small amounts of denatured protein that slowly form at low temperatures. It was then diluted with an equal volume of 0.2 *M* phosphate buffer, pH 7.05 at room temperature. The resulting solution, containing 6 mg. TMV/cc., was used within not more than 4 hrs. Stainless steel bombs, illustrated by the diagram in Fig. 1, were filled with approximately 3 cc. portions and connected to the hydraulic pumps or to a "dummy" connection. The bombs were preheated in a water bath at 60°C. for 3 mins., then transferred at once to the 68.8 or the 72.5°C. bath for the desired period of time. Cooling was accomplished quickly in a water bath at room temperature. No detectable denaturation occurred during the preheating, which served to reduce the lag and minimize the influence of changing rates of reaction in reaching the higher temperature. The kinetics, in fact, indicated no clear evidence of a lag, but rather a very brief initial period of more rapid denaturation, at 68.8°C., as described below. In all experiments at increased pressure, the pressure was applied before heating and was released after cooling.

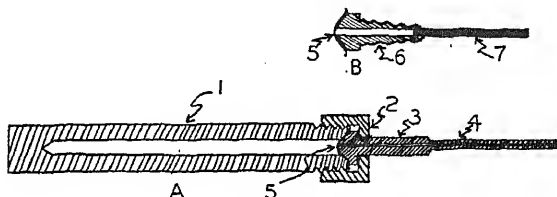


FIG. 1. Diagram of apparatus for thermal denaturation of the virus solution under increased pressure (A), and type of closure used in experiments at normal pressure (B). A stainless steel "bomb" (1) outer diameter 7/16", capacity about 3 cc., is filled with virus solution and a disc of rubber dental dam (5) placed over the opening. The fitting (3) of the oil pressure line (4) from the hydraulic pump is then attached by nut (2), and pressure applied. The pressure line consists of copper tubing, 0.03" inner diameter, and 0.125" outer diameter. For normal pressure, a dummy closure (B), made from the brass hose connection (b) of a 1/8" I. P. Hoke needle valve, is used. The virus solution is separated from the closure again by dental dam (5). A brass rod (7) is soldered to the opposite end, leaving an air space between. The air space is important, since it allows the virus solution to expand under heat, whereas if the solution were confined within a completely rigid container, pressures of as much as 1000 lb./in² are built up when the temperature of the solution is raised from room temperature to 70°C.

Following heat treatment, the specimen was centrifuged for 10 mins. at 12,700 *g*

The precipitate was discarded, and the supernatant analyzed for protein remaining in solution by micro Kjeldahl. Corrections were made according to the formula of Lauffer and Price (1) for the nucleic acid that splits off and remains in solution.

RESULTS

Although the kinetics and rate constants of denaturation at normal pressure were found to conform in general with the results reported by

Lauffer and Price (1),³ two peculiarities were observed in connection with brief times of heating at 68.8°C.

First, solutions only slightly denatured (*e.g.*, after 10 mins. at 68.8°C. at normal pressure) contained, in addition to a precipitate that could be readily separated from the undenatured virus by centrifugation at 10,000–12,000 *g*, another fraction which contributed to opacity but which remained in the supernatant. At higher forces, of about 17,000 *g*, both this fraction and the native virus sedimented at essentially the same rate. In all cases, however, it constituted only a small portion of insoluble the protein, and it was not observed after centrifugation of the more fully denatured solutions. Consequently, it gave rise at most to only a small error.

A second peculiarity, noted at 68.8° but not at 72.5°C., consisted in a rapid initial fall in soluble protein, with the result that during the first 5–10 mins. at normal pressure, the kinetics of precipitation were not the same as during the first order rate process which followed. The cause of this phenomenon, again, is not clear. Reproducible results were obtained by keeping the stock virus solution in distilled water at refrigerator temperatures, then diluting in phosphate buffer just before the experiment.

Apart from the two complications just referred to, the denaturation of the virus, as measured by the method described, proceeds in accordance with the kinetics of a first order rate process. Thus, although more than one reaction may be involved in the change from the native, soluble to the denatured, insoluble form which precipitates, evidently one single reaction is predominantly rate-determining. The results with hydrostatic pressure provide further evidence that this is the case.

³ A portion of the same virus preparation that was used in this study was subsequently stored, in a more concentrated solution of about 30 mg. protein/cc. in distilled water, for 4 months in the refrigerator. It was then repurified in the ultracentrifuge and found to be highly aggregated as judged by viscosity and sedimentation characteristics. The rate of denaturation at 68.8°C. was also different, though not entirely because of the aggregation. We have since found that there may be considerable variation, under similar conditions, in the rate of denaturation of different specimens of freshly isolated virus preparations. The cause for such variations is not yet clear but the fact is mentioned to avoid having any unjustified significance attached to the fairly close agreement between the numerical values of the rate constants at normal, pressure reported herewith and those given earlier by Lauffer and Price (1).

According to the theory of Absolute Reaction Rates (7), the specific reaction rate, k' , is given by the expression

$$k' = \kappa \frac{kT}{h} K^\ddagger, \quad (1)$$

in which the term $\kappa \frac{kT}{h}$ represents the universal frequency for the decomposition of the activated complex in all chemical reactions; κ is the transmission coefficient (usually equal to 1), k the Boltzmann constant, T the Absolute temperature, and h is Planck's constant. The constant K^\ddagger is the equilibrium constant between the normal and activated state of the reactants and is expressed in the same manner as an ordinary equilibrium constant:

$$K^\ddagger = e^{-(\Delta F^\ddagger/RT)} = e^{-(\Delta H^\ddagger - T\Delta S^\ddagger)/RT} = e^{-(\Delta E^\ddagger + p\Delta V^\ddagger - T\Delta S^\ddagger)/RT}, \quad (2)$$

where ΔF^\ddagger refers to the free energy, ΔS^\ddagger the entropy, ΔV^\ddagger the volume change, ΔH^\ddagger the heat, and ΔE^\ddagger the energy, of activation, rather than of reaction. The other symbols have the usual meaning: T , the absolute temperature; R , the gas constant (82.07 cc.); and p the pressure. From Eqs. (1) and (2) it is apparent that, knowing the numerical values of the constants, ΔF^\ddagger , ΔH^\ddagger , ΔV^\ddagger , etc., it should be possible to calculate the rate of the reaction at any temperature and at any pressure, provided the mechanism of the reaction remains the same. The work of Lauffer and Price (1) has indicated a value of 153,000 calories for ΔH^\ddagger at pH 7.0, and ΔV^\ddagger can be computed from the rates of denaturation at a given temperature under two different pressures, making use of the expression,

$$\Delta V^\ddagger = RT \frac{\ln k_{p_1} - \ln k_{p_2}}{p_2 - p_1}. \quad (3)$$

Agreement with the theory may be judged by the success with which the rates at different temperatures and pressures are quantitatively predicted with the aid of these equations. The results of the experiments have been found to conform closely with the theory, as discussed in the following paragraphs.

In undertaking to obtain the numerical value of ΔV^\ddagger , a series of experiments was first carried out in which the amount of denatured protein was determined gravimetrically. Four cc. portions of an old virus solution, initially containing about 6 mg./cc., were introduced into small, tared test tubes previously selected to contain just this volume of solution. Each tube was closed by means of a double layer of rubber dental dam, held in place by tight rubber bands. The tubes were immersed in water in a steel pressure chamber, which was then heated, under normal or under 9600 lbs./in.² pressure, in a water bath for the desired length of time, varying from 15 mins. to 7 hrs. Following the heat treatment, the tubes were centrifuged in an ordinary clinical centrifuge, the supernatant decanted, and the precipitate washed with distilled water. The precipitate was then dried to constant weight at 70°C. Similar tubes were heated for 30 mins. at 100°C. to bring about complete denaturation, and to determine the total precipitable protein. The logarithm of the per cent of this total that was precipitated after various intervals of heating at 68.8°C. under normal and increased pressure was plotted against time. Straight lines resulted, and the first order rate constants for the two pressures were computed. The data, substituted in Eq. (3) gave a value for ΔV^\ddagger of 100.07 cc./mole.

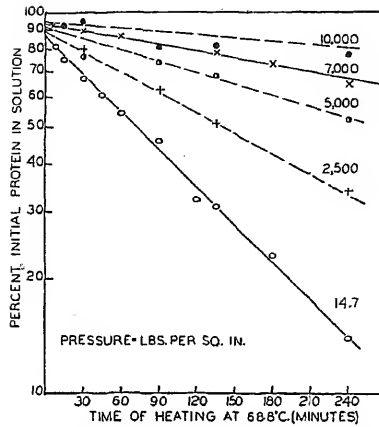


FIG. 2. Rate of denaturation by TMV protein, in terms of the amount of protein remaining in solution, as a function of hydrostatic pressure at 68.8°C. The volume change of activation, 100 cc./mole, was computed from the observed rates at normal and 7000 lbs./in.² pressure (solid lines). The rates at other pressures (broken lines) were then calculated on the basis of the theory of Absolute Reaction Rates, assuming a single first order rate process as a rate-determining reaction. The points represent data from experiments.

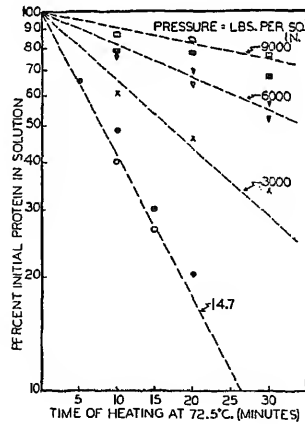


FIG. 3. Calculated rates (broken lines) of denaturation of TMV protein at 72.5°C., according to a volume increase of activation of 100 cc./mole, and activation energy of 153,000 cal. The solid and hollow points represent data individual determinations of protein nitrogen in solution in repeated experiments.

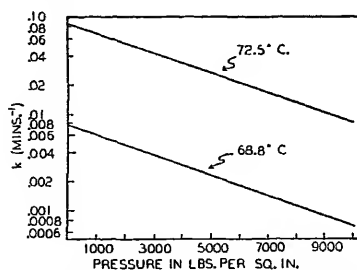


FIG. 4. The first order rate constant (k) calculated as a function of pressure at two temperatures, according to the Theory of Absolute Reaction Rates, with 100 cc. as the value for ΔV^\ddagger , and 153,000 cal. for ΔH^\ddagger .

In the remaining experiments, the amount of denaturation was measured in terms of protein remaining in solution according to the method described. The first order rate constants at normal and at 7000 lbs./in.², respectively, were first determined. The data are shown in Fig. 2 (solid lines). From these constants, 0.007594 min.⁻¹ at normal, and 0.001366 min.⁻¹ at 7000 lbs. pressure, ΔV^\ddagger was computed to be 101.26 cc., in satisfactory agreement with the value arrived at through the gravimetric method of measuring denaturation.

Using 100 cc./mole for ΔV^\ddagger , in round numbers, and using the figure of 153,000 calories given by Lauffer and Price (1) for ΔH^\ddagger , the rates of precipitation at both 68.8 and 72.5°C., under various pressures, were now calculated. The calculated rates are

TABLE I
*Physical Constants for the Thermal Denaturation
of Tobacco Mosaic Virus at pH 7.0.*

Temp.	Pressure	First order rate constant		ΔF^\ddagger	ΔH^\ddagger	ΔS^\ddagger	ΔV^\ddagger
		min. ⁻¹	sec. ⁻¹				
°C.	(atm.)			cal.	cal.	cal./degree	cc./mole
68.8	1	.007594 ^a	.0001265 ^a	26,200	153,000 ^b	370.89	100.
68.8	170.	.004158	.0000693	26,609	153,000 ^b	369.67	100.
68.8	340.1	.002266	.00003776	27,021	153,000 ^b	369.67	100.
68.8	476.2	.001366 ^a	.00002277	27,365	153,000 ^b	367.46	100.
68.8	680.3	.000674	.00001124	27,844	153,000 ^b	366.06	100.
72.5	1	.08660	.0014433	24,819	153,000 ^b	370.89	100.
72.5	204.8	.04135	.0006891	25,327	153,000 ^b	369.42	100.
72.5	408.16	.01996	.0003326	25,827	153,000 ^b	367.98	100.
72.5	612.24	.00971	.0001619	26,322	153,000 ^b	366.55	100.

^a Determined by experiments, this study. All other data calculated (except ΔH^\ddagger).

^b Figure reported by Lauffer and Price (1).

indicated by the broken lines in Figs. 2 and 3. At the lower temperature (Fig. 2), the intercept at 0 time was predicted, semiempirically, by assuming that the logarithm of the intercept would be proportional to pressure, and using the intercepts that were found by extrapolating back to zero time the straight-lines obtained for precipitation at normal and 7000 lbs. pressure (Fig. 2). For the higher temperature (Fig. 3), no initial, more rapid denaturation was assumed. In Figs. 2 and 3 many of the points obtained from the experiments fall either on, or very close to, the previously calculated lines. Discrepancies are within an experimental error of $\pm 5\%$, except at the highest pressures, where the total amount of denaturation was relatively slight and the error somewhat greater. These results indicate satisfactory agreement with the theory assumed. The calculated rate constants as a function of pressure, at the two temperatures, are plotted in Fig. 4, and numerical data are summarized in Table I.

DISCUSSION

It is worthy of note that the rates of TMV denaturation at various pressures may be as successfully predicted, on the basis of the Theory of Absolute Reaction Rates with the appropriate numerical value of the volume change of activation, ΔV^\ddagger , as the rates at different temperatures, with the appropriate value of the activation energy, ΔH^\ddagger . The agreement between the calculated and experimental data was close, justifying the use of the formulation for a single reaction as rate-determining.

With regard to the volume change itself, 100 cc./mole is of the same order of magnitude as in the inactivation of invertase (8), the denaturation of human serum globulin (9), and the specific precipitation of rabbit antibody (10). Yet TMV is a vastly larger molecule than these other proteins, and in spite of the greater number of bonds that might be broken, it would appear that the limiting reaction may involve relatively few of them. Moreover, the activation energies and entropy changes in the thermal denaturation of TMV are also of the same magnitude as in a number of much smaller molecules, such as egg albumin, hemoglobin, and others (*cf.* Eyring and Stearn, 11). Thus, possibly the same types, as well as approximately the same number of bonds, are somewhat generally concerned in the denaturation reaction.

A volume increase of this magnitude in proteins, although only a small fraction of the total volume, especially in TMV where it amounts to about 4 parts per million, has been interpreted as representing a very pronounced change in structural configuration (8), perhaps through an unfolding from a somewhat globular to a more linear form. Various agents, including alcohol, urethane, hydroxyl ions, *etc.*, catalyze a reversible reaction with volume change of this sort in certain proteins,

such as the luminescent enzyme of bacteria, and it has been postulated that a similar unfolding must precede the reproduction of virus molecules in living host tissues (8) by a templet mechanism. Consequently, it would be anticipated that pressure should be as effective in opposing the propagation as the denaturation of the virus.

The relation between the denaturation reaction which is retarded by pressures of less than 1000 atm. at around 70°C., and the one which is accelerated by pressures between 5000 and 10,000 atm. at 30°C. remains to be clarified. The fact that the influence of pressure is in opposite directions shows that the mechanism of denaturation is not the same in the two cases. For the latter, *i.e.*, the denaturation of proteins at ordinary temperatures by very high pressures, no clear interpretation has been available, but Eyring has suggested ⁴ that this reaction probably accompanies a change in the structure of the solvent, as follows.

It is known that the structure of water varies with pressure as well as temperature (12); for example, at a little above 2000 atm., ice III, which is some 10% denser than water, becomes stable as compared with ice I, which is about 10% less dense than water, and moreover, ice II, III, V, and VI are all denser than water. The native form of the protein is a highly ordered structure as shown by the great increase in entropy on denaturation. A radical crushing of the solvent would be expected to lessen the stability of the highly ordered native protein, with the result that it breaks down following the change in solvent structure. In connection with this hypothesis, data concerning the relation between temperature and the denaturation of proteins under very high pressures would be interesting. Although such data are largely lacking, there is suggestive evidence that the reaction may have a small negative temperature coefficient since Bridgman (13) has observed that, in the coagulation of egg albumen under 6000 atm. pressure, a somewhat greater stiffening was caused in one hr. at 0°C. than at 20°C.

Large differences in activation energies and volume changes would be sufficient to account for one reaction rather than another to predominate under different conditions of temperature and pressure within the range of 30°–70°C., and 680–6000 atms., respectively.⁵

⁴ Personal communication from Dean Henry Eyring of the Graduate School, Univ. of Utah.

⁵ We have found, in fact, through statistical analyses of a number of more recent parallel experiments that the numerical value of ΔV^\ddagger tends to decrease slightly at the higher pressures within this range, up to 680 atm.

ACKNOWLEDGMENT

The authors are indebted to Miss Virginia K. Miller for her capable technical assistance throughout this investigation.

SUMMARY

Thermal denaturation of tobacco mosaic virus protein in phosphate buffer, pH 7.05, at 68.8 and $72.5 \pm 0.02^\circ\text{C}$. is retarded by hydrostatic pressures up to 680 atm. The volume increase of activation, ΔV^\ddagger , computed from the observed first order rate constants at two pressures and 68.8°C ., amounted to very close to 100 cc./mole. Using this figure for ΔV^\ddagger , and 153,000 cal. for ΔH^\ddagger reported by Lauffer and Price (1), the specific rates at various pressures and the two temperatures were calculated on the basis of the theory of Absolute Reaction Rates and found to agree closely with the data from experiments. The free energy of activation was calculated to be 26,200 at 68.8°C ., and 24,819 at 72.5°C ., respectively, with the entropy of activation, at normal pressure and these temperatures, as 370.89 E. U.

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Studies of Thyroid Toxicity. II. The Effects of Desiccated Thyroid and Anti-Thyroid Agents upon the Plasma and Tissue Ascorbic Acid of Rabbits¹

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INTRODUCTION

Many reports have appeared in the literature concerning the interrelationships between the thyroid hormone and ascorbic acid in species known to require a dietary source of this vitamin. In general, the results of these studies have indicated an antagonism between this vitamin and the thyroid hormone. Thaddea and Scharlach (2) found that the weight loss and shortened life span of guinea pigs, given thyroxine on a normal or scorbutic diet, could be alleviated by the administration of ascorbic acid. This was confirmed in part by Wilhelmi (3). The effects of thyradin, or thyroid powder, can be suppressed by the simultaneous injection of ascorbic acid according to Nisino and Kozyōma (4). Thaddea and coworkers also noted that thyroidectomy of either normal or scorbutic guinea pigs (2) or normal dogs (5) caused an increase in the ascorbic acid content of the liver and adrenals. Administration of thyroxine, or thyrotropic hormone, caused a decrease in the ascorbic acid content of the tissues which could be mitigated by the administration of ascorbic acid. The work of Monetti (6) and of Ghosh (7) also indicated that the administration of thyroxine to guinea pigs caused a decrease in the ascorbic acid content of the lungs, kidneys, and adrenals. It has also been noted that the injection of hormones other than that of the thyroid gland alters the plasma ascorbic acid of animals (see 8).

Much of the previous work has been carried on with guinea pigs, which require an exogenous source of ascorbic acid.³ The present experiments were designed to study the relationship of hypo- and hyper-

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² Wisconsin Alumni Research Foundation Fellow.

³ For a review of the literature see Drill (9). In the single reference by Drill to work done with rabbits [Ray, S. N., *J. Ind. Chem. Soc.* 15, 237 (1938)] no mention is made of this animal. Ray worked with guinea pigs and rats.

thyroidism to the metabolism of ascorbic acid in the rabbit, a species which is known to synthesize amounts of this vitamin adequate to meet its normal requirements. Desiccated thyroid was used to obtain a condition of hyperthyroidism; thiourea, thiouracil, and propylthiouracil were used to achieve the effects of hypothyroidism by inhibiting the synthesis of the thyroid hormone.

EXPERIMENTAL

The rabbits were kept in metal cages with screen floors and maintained on a commercial pellet ration and water. Desiccated thyroid, thiourea, thiouracil, or propylthiouracil was given daily by capsule during the experimental period. The control animals were given empty capsules. Blood samples were collected in oxalated tubes from a cut made in the marginal vein of the ear. Blood plasma reduced ascorbic acid was determined by the method of Mindlin and Butler (10), and total ascorbic acid by the method of Roe and Kuether (11). Ascorbic acid analyses were made prior to the experimental period to obtain normal values, and during the treatment period to follow the effect of the drugs. At the end of the treatment period some control and experimental animals were sacrificed and reduced ascorbic acid analyses made on certain of their tissues. The animals were killed by exsanguination, the organs to be analyzed were removed immediately and placed in chipped ice. Tissue samples were homogenized in 6% HPO_3 and filtered. The extracts were made up to the proper dilution, treated with 2,6-dichlorophenolindophenol and read in an Evelyn colorimeter with a 520 $\text{m}\mu$ filter in 30 sec. Rabbits of the same age and strain, and with similar nutritional histories, were used in each experiment.

RESULTS

Variation of Blood Plasma Ascorbic Acid with Age

Most of the experiments reported here were performed with rabbits which had almost reached maturity. Control animals of this age maintained relatively uniform levels of plasma ascorbic acid (0.25–0.5 mg.—%). In those experiments performed with young rabbits (1.5–2.0 kg.) initial values for plasma ascorbic acid were much higher (0.7–1.0 mg.—%) and control animals showed a gradual decrease as they approached maturity. Fig. 1 illustrates the decrease in plasma reduced ascorbic acid which occurred in the control rabbits from Exp. A below.

Influence of Desiccated Thyroid on the Ascorbic Acid of Growing Rabbits

The effect of various doses of desiccated thyroid upon the plasma and tissue reduced ascorbic acid was studied. It can be seen (Table I) that

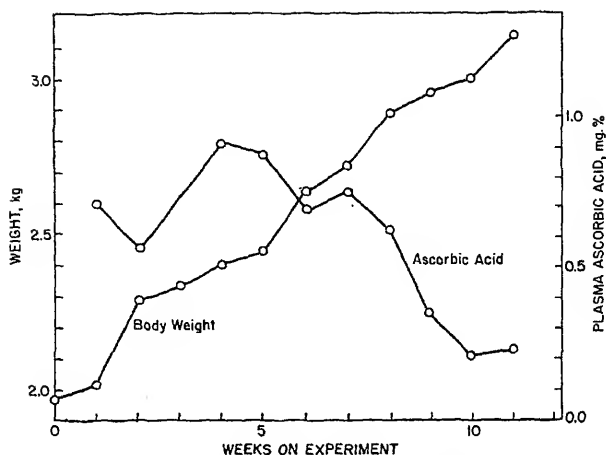


FIG. 1. Growth curve of 2 control rabbits and accompanying decrease in their plasma reduced ascorbic acid.

TABLE I
Effect of Desiccated Thyroid on Plasma Reduced Ascorbic Acid

Dosage	No. of rabbits	Plasma reduced ascorbic acid					
		Pre-exp. ^d	1 week	2 week	3 week	4 week	5 week
Experiment A ^a		mg.—%					
Control	2	0.72	0.87	0.69	0.74	0.62	0.35
50 mg./kg./day	3	0.52	0.14	0.14	0.14	0.10	0.01 ^e
70 mg./kg./day	3	0.37		0.38	0.23		0.06 ^f
Experiment B ^b							
Control	3	0.33	0.41	0.40	0.44	0.42	
25 mg./kg./day	1	0.35	0.04 ^e				
15 mg./kg./day	1	0.25	0.13	0.03 ^e			
8 mg./kg./day	2	0.33	0.39	0.20	0.24	0.20 ^f	

^a Wilson USP desiccated thyroid.

^b Parke-Davis desiccated thyroid.

^c One died during treatment period.

^d Preexp. = average of determinations made prior to treatment.

^e Died during treatment period.

^f Sacrificed and used for tissue analysis.

the plasma reduced ascorbic acid was decreased by oral administration of the proper dosage of desiccated thyroid. The greater survival of the rabbits in Exp. A is due chiefly to differences in the activity of the preparations used. The desiccated thyroid used in Exp. B was of greater potency and caused the death of several of the rabbits. Rabbits fed desiccated thyroid suffered an immediate weight loss and displayed symptoms of hyperirritability, but appeared normal in other respects. Animals fed light doses slowly recovered the lost weight, while those

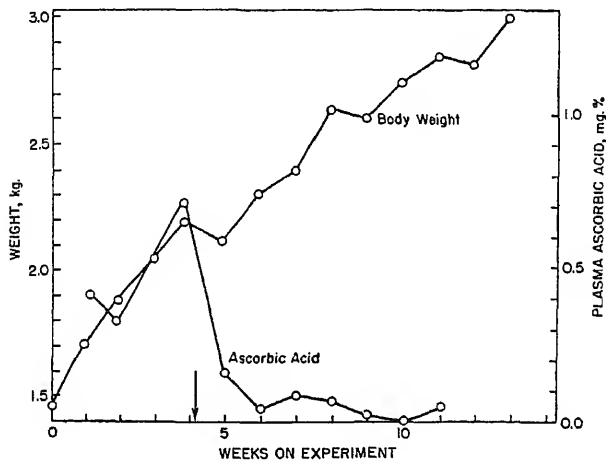


FIG. 2. Apparent normal growth rate of a rabbit depleted of ascorbic acid. Administration of desiccated thyroid (50 mg./kg./day) begun at arrow. Growth of control rabbits is shown in Fig. 1.

given the larger doses continued to lose weight, stopped eating, and eventually succumbed. Some individuals, whose resistance to desiccated thyroid was apparently greater than average, continued to grow at almost a normal rate even though their plasma reduced ascorbic acid was extremely low (Fig. 2). The results of tissue analyses made on some rabbits which received the desiccated thyroid preparation used in Exp. A are shown in Table II. The treatment caused a decrease in the ascorbic acid content of all the tissues analyzed except the brain, which remained remarkably constant. The spinal fluid and blood plasma ascorbic acid were elevated above normal by oral ascorbic acid supplementation, even when the latter was given with desiccated thyroid.

TABLE II
*Effect of Desiccated Thyroid and Ascorbic Acid on Tissue
 Ascorbic Acid*

Dosage ^a	No. of animals	Ascorbic acid content of various tissues			
		Adrenal	Brain	Spinal fluid	Blood plasma
		<i>mg./gland</i>	<i>mg./g.</i>	<i>mg.—%</i>	<i>mg.—%</i>
Control	3	0.43	0.14	1.26	0.24
Desiccated thyroid ^b	3	0.10	0.16	0.26	0.09
70 mg./kg./day					
Ascorbic acid 50 mg./kg./day	1	0.35	0.14	2.04	0.81
Desiccated thyroid + ascorbic acid (same levels as used above)	2	0.36	0.15	1.80	0.82

^a Treatment continued for 33 days.

^b Wilson USP desiccated thyroid.

The adrenal ascorbic acid content, however, was not elevated above normal by this treatment.

Data on ascorbic acid were also obtained from another experiment in which 10 young rabbits were injected with DL-thyroxine at the rate of 0.5 mg./kg. body weight daily for one week. Blood plasma collected on the seventh day contained only traces of ascorbic acid. Thereafter, the rabbits were given 0.5 mg. thyroxine/kg. body weight every third day. Blood samples collected at weekly intervals contained no detectable amounts of ascorbic acid as long as the treatment with thyroxine

TABLE III
Effect of Thiourea on Plasma Reduced Ascorbic Acid

Dosage	No. of rabbits	Plasma reduced ascorbic acid			
		Pre-exp. ^a	1 week	2 weeks	3 weeks
			<i>mg.—%</i>		
None (control)	3	0.33	0.41	0.40	0.44
1.71 mM/kg./day	4	0.29	0.01	0.00	0.00
1.31 mM/kg./day	2	0.24	0.03	0.03	0.00
0.4 mM/kg./day	4	0.33	0.03	0.07	0.01
0.13 mM/kg./day	3	0.38	0.10	0.11	0.12

^a Preexp. = average of determinations made prior to treatment.

continued. The plasma reduced ascorbic acid of the control rabbits varied between 0.62 and 0.20 mg.—% during this period.

Action of Antithyroid Drugs on the Ascorbic Acid of Adult Rabbits

Following the confirmation that desiccated thyroid or thyroxine decreased the tissue concentration of ascorbic acid, it became of interest to study the effect of eliminating the endogenous production of thyroid

TABLE IV
Effect of Thiourea on Total and Reduced Plasma Ascorbic Acid

No. of rabbits	Dosage	Analysis	Plasma ascorbic acid						
			Pre-exp. ^a	2 days	5 days	1 week	2 weeks	3 weeks	4 weeks
4	0.4 mM thiourea/kg./day	Reduced ascorbic acid	0.33	0.12	0.04	0.03	0.07	0.01	0.02
		Total ascorbic acid	0.40	0.27	0.11	0.11	0.11	0.11	0.08
2	Control	Reduced ascorbic acid	0.23		0.21	0.13	0.12	0.15	0.22
		Total ascorbic acid	0.27		0.28	0.23	0.28	0.27	0.33

^a Preexp. = average of determinations made prior to treatment.

hormone. Thiourea was first used to achieve this effect. Contrary to expectations it was found that this antithyroid agent also depressed the level of plasma ascorbic acid (Table III). Doses as small as 10 mg. (0.13 mM)/kg. body weight/day reduced the plasma concentration of ascorbic acid to $\frac{1}{4}$ the preexperimental level. Larger doses resulted in complete disappearance of detectable quantities of the vitamin from the blood plasma. Nearly all the animals given thiourea displayed a

weight loss of approximately 10% immediately after treatment was started. Thereafter, the weight fluctuated about this lowered level.

That the plasma ascorbic acid is truly lowered and not converted into an oxidized form which is not detected by the method of Mindlin and Butler (10) is demonstrated by the data in Table IV. The amount of "dehydroascorbic acid" present was no greater in the rabbits fed thiourea than in the controls.

Data in Table IV show that following the administration of thiourea a decrease in plasma ascorbic acid occurred within 2 days. To obtain more data on the speed of this decrease, one rabbit was given a large

TABLE V
Effect of Thiouracil on Plasma Reduced Ascorbic Acid

Dosage	No. of rabbits	Plasma reduced ascorbic acid				
		Pre-exp. ^a	1 week	2 weeks	3 weeks	4 weeks
None (control)	3	0.33	0.41	mg.—% 0.40	0.44	0.49
1.16 mM/kg./day	1	0.43	0.11	0.16	0.15	0.16
0.63 mM/kg./day	1	0.18	0.08	0.14	0.21	0.11
0.59 mM/kg./day	1	0.37	0.07	0.11	0.18	0.24
0.39 mM/kg./day	2	0.38	0.30	0.31	0.37	
0.08 mM/kg./day	3	0.46	0.38	0.36	0.48	

^a Preexp. = average of determinations made prior to treatment.

dose of thiourea (1.0 g.) and its plasma reduced ascorbic acid was determined at 2-hr. intervals for 12 hr. No significant decrease was observed in that period but at 24 hr. the level was approximately $\frac{1}{3}$ of the original value.

The possibility of thiourea reacting with ascorbic acid to form a compound which is not detected by the method of Mindlin and Butler (10) was investigated *in vitro*. No evidence could be found that thiourea combined with ascorbic acid or prevented its reducing the indophenol dye. However, these results do not eliminate the possibility of such a reaction taking place *in vivo*. Urinary ascorbic acid analyses made on several of the rabbits which received thiourea did not show a higher than normal excretion of ascorbic acid during this treatment.

Table V displays the effect of various doses of thiouracil upon the

plasma reduced ascorbic acid. The effect of this drug is similar, in that a reduction of the plasma ascorbic acid occurs. It is to be noted that thiouracil did not lower the level of ascorbic acid as far as did thiourea and that, after falling, the level rose again in spite of continued treatment. However, in the case of the rabbit which received the largest dose (150 mg. (1.16 mM)/kg. body wt./day) a low level of ascorbic acid was maintained throughout the 4 week experimental period.

Propylthiouracil, fed at doses up to 200 mg. (1.17 mM)/kg. daily caused no alteration of the plasma reduced ascorbic acid (Table VI).

TABLE VI
Effect of Propylthiouracil on Plasma Reduced Ascorbic Acid

Dosage	No. of rabbits	Plasma reduced ascorbic acid					
		Pre-exp ^a	2 days	7 days	10 days	14 days	17 days
Control	3	0.33	0.28	0.33	0.29	0.29	0.32
0.23 mM propylthiouracil/kg./day	3	0.40	0.54	0.54	0.68	0.66	0.50
0.59 mM propylthiouracil/kg./day	3	0.36	0.22	0.47	0.29	0.37	0.38
1.16 mM propylthiouracil/kg./day	3	0.29	0.32	0.34	0.43	0.41	0.30

^a Preexp. = average of determinations made prior to treatment.

Thiourea and thiouracil also have a depleting action upon the ascorbic acid content of the tissues (Table VII). The administration of thiourea decreased the concentration of ascorbic acid in adrenal, brain, liver, and testicular tissues. It was noted that the reduction of adrenal ascorbic acid was proportional to the dose of thiourea administered. The brain, liver, and testicle ascorbic acid was reduced to the same amount regardless of the dose. Thiouracil likewise caused a decrease in the tissue concentration of ascorbic acid but it was much less effective than thiourea. At doses of 50 mg. (0.39 mM)/kg./day, or less, thiouracil did not affect either the blood plasma or tissue concentration of vitamin C.

The depleting action of thiourea on tissue ascorbic acid seemed to offer an excellent tool for the production of experimental scurvy in a species which ordinarily requires no dietary source of vitamin C. To

test this possibility 3 rabbits were given heavy doses of thiourea (200 mg. (1.62 mM)/kg./day) for an extended period of time. At the end of 4 months of treatment the animals appeared somewhat lethargic, although otherwise normal. Their plasma ascorbic acid fluctuated between 0.0 and 0.1 mg.—% during this time. One rabbit apparently developed a resistance to the drug. Its plasma reduced ascorbic acid rose and approached the preexperimental average (0.42 mg.—%) even though treatment continued. The other 2 rabbits were taken off treat-

TABLE VII
Effect of Anti-Thyroid Drugs on Tissue Reduced Ascorbic Acid

Treatment	No. of rabbits	Days on treatment	Ascorbic acid content of various tissues			
			Adrenal	Brain	Liver	Testicle
None (control)	2	21	1.6	0.16	0.13	0.20
1.31 mM thiourea/kg./day	2	21	0.3	0.06	0.03	Not analyzed
0.66 mM thiourea/kg./day	2	30	0.50	0.05	0.03	0.06
0.4 mM thiourea/kg./day	1	30	0.6	0.05	0.03	0.06
1.16 mM thiouracil/kg./day	1	28	0.7	0.12	0.08	0.14
0.63 mM thiouracil/kg./day	1	30	1.2	0.11	0.05	0.12
0.59 mM thiouracil/kg./day	1	28	1.6	0.15	0.10	0.17
0.39 mM thiouracil/kg./day	1	21	1.7	0.16	0.14	Not analyzed

ment at the end of 4 months. One of these animals survived for a month following termination of treatment. However, during this month no plasma reduced ascorbic acid could be detected. The animal displayed anorexia, cachexia and lethargy before death. The second animal responded somewhat differently when taken off treatment. Its plasma ascorbic acid and weight rose immediately but the animal died within 5 days.

DISCUSSION

The finding that the ingestion of desiccated thyroid substance depresses the blood and tissue ascorbic acid concentration in rabbits is in agreement with the work which has been done with guinea pigs and rats (9).

Thyroidectomy has been reported to increase the ascorbic acid content of animal tissues. It might, therefore, be expected that agents which prevent the endogenous production of thyroxin would likewise

cause an increase in the tissue level of ascorbic acid. Contrary to this, an abrupt decrease in the plasma ascorbic acid was observed following the feeding of thiourea or thiouracil to rabbits.

The experimental results here reported bring out two points which merit further consideration. First, it is apparent that, in the growing rabbit, blood plasma ascorbic acid levels may be kept at extremely low levels (either by thyroid hormone or antithyroid agents) without seriously impairing growth of the animals and without the appearance of any symptoms of scurvy.

Secondly, the results point to the possibility that some of the toxic effects resulting from the clinical use of thiourea and thiouracil may be connected with its depleting action on tissue ascorbic acid. It is recognized that, on a body weight basis, the doses of these agents given to rabbits are many times greater than used in clinical practice. Nevertheless, the prolonged ingestion of smaller doses by a species not capable of synthesizing its own supply of ascorbic acid might lead to similar results. Propylthiouracil which, because of its lesser toxicity, has almost completely replaced the other two agents in clinical practice, was found to have no depleting effect on the blood plasma ascorbic acid.

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SUMMARY

1. The plasma ascorbic acid content of young rabbits is high and decreases as the animal matures.
2. In confirmation of previous work with other species the ingestion of desiccated thyroid by rabbits caused a marked reduction of plasma and tissue ascorbic acid levels.
3. Certain rabbits appeared to be more resistant to the harmful effects of desiccated thyroid, and when proper doses were administered to these young animals growth was maintained coincident with very low levels of plasma ascorbic acid.
4. Thiourea caused an extreme reduction of the plasma and tissue reduced ascorbic acid.
5. Feeding thiouracil also reduced the ascorbic acid content of rabbits' plasma and tissues but not as effectively as did thiourea.

6. When minimally effective doses of thiouracil were given, the plasma ascorbic acid rose again after the initial reduction, even though treatment continued.

7. Propylthiouracil produced no significant alteration in the plasma ascorbic acid level.

8. When given high, but not lethal, amounts of thiourea, rabbits will live for long periods of time with an extremely low concentration of plasma ascorbic acid without the development of any symptoms of scurvy.

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The Accumulation of Acid-Labile, Inorganic Phosphate by Mutants of *Neurospora*¹

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INTRODUCTION

The occurrence of metaphosphate has been reported in yeast (9, 10, 16, 18, 19), and in *Aspergillus niger* (11). Wiame states that this compound is a normal constituent of yeast cells and that abnormally large amounts are found under certain culture conditions.

It is the purpose of this paper to describe the isolation of a similar compound from *Neurospora*. The structure of this compound is not known, nor is it known whether it is identical with the metaphosphate found in yeast and *A. niger*. The fact that it has been isolated from wild-type *Neurospora* indicates that it is a normal constituent. The point of interest, however, is that it is accumulated by 4 genetically and physiologically distinct mutant strains. One of these strains, under appropriate conditions of growth, accumulates as much as 10 times the quantity found in the wild-type.

EXPERIMENTAL

Preparation of Trichloroacetic Acid Extracts

In most experiments mycelium was obtained from cultures in 125 ml. Erlenmeyer flasks containing 20 ml. of basal medium (1) appropriately supplemented. In a few experiments mycelium was produced under forced aeration in 5-gal. bottles containing 15 l. of medium. At the end of the growth period, usually 4 days, the medium was filtered off and the mold pressed as nearly free of medium as possible. It was then quickly ground in a mortar with pulverized glass in ice-cold 10% trichloroacetic acid solution (5 or 10 ml./g. of mold). The mixture was transferred to centrifuge tubes, kept in an ice bath for one hour and then centrifuged.

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Determination of Phosphate

Orthophosphate was determined in one sample of this extract without further treatment. A second sample was heated with an equal volume of 2 *N* HCl for 10 mins. at 100°C., and cooled in an ice bath before the determination. Determinations were made colorimetrically using ferrous sulfate as the reducing agent. The color was measured by means of a Beckman spectrophotometer at wave length 6250 Å.

Strains Tested

Some of the strains of *Neurospora* which have been tested in the above fashion are listed in Table I. It will be seen that abnormally high values for acid-labile phosphate, which is hydrolyzable under the conditions given above, were obtained for 5 mutant strains, pyrimidineless 37301 (13), lysineless 4545 (6), nicotinicless 39401 and 65001,

TABLE I
Acid-Labile Phosphate in Trichloroacetic Extracts

Strain number	Requirement	Supplement mg./20 ml.	Acid-labile PO ₄ mg./g. wet wt.
263	Pyrimidine	Cytidine sulfate 0.5	1.4
37301	Pyrimidine	Cytidine sulfate 0.5	6.3
38502	Pyrimidine	Cytidine sulfate 0.5	1.5
4545	Lysine	L(+)-Lysine mono- hydrochloride 1.0	6.8
44409	Unknown	None	1.3
45208	Unknown	None	0.9
55701	Unknown	None	4.9
80904	Unknown	None	1.1
83106	Unknown	None	1.5
Abbott 4A	Wild	None	1.1
Abbott 12A	Wild	None	1.0
Emerson 5256A	Wild	None	1.4
Emerson 5297a	Wild	None	1.7
1A	Wild	None	1.4
25a	Wild	None	1.6
Chilton a	Wild	None	1.5
B 1167	Adenine	Adenine sulfate 1.0	1.6
27663	Adenine	Adenine sulfate 1.0	1.1
28610	Adenine	Adenine sulfate 1.0	1.9
35203	Adenine	Adenine sulfate 1.0	1.3
44206	Adenine	Adenine sulfate 1.0	2.0
70004	Adenine	Adenine sulfate 1.0	2.5
39401	Nicotinic	L-Kynurenine 0.05	22.5
65001	Nicotinic	L-Kynurenine 0.05	13.2

(2, 4), and 55701, unknown requirement, not previously described. (The two nicotinicless strains are probably alleles.) Strain 55701 grows slowly at 25°C. on the basal medium but is markedly stimulated by pyruvate or acetate. At 35°C. it does not grow on any medium which has been tried. (Four other strains described in the table as having unknown requirements, although genetically different from 55701, show the same growth response at 25°C and 35°C. but are not affected by pyruvate or acetate.) Reisolated strains of 4545, 37301, and 65001 were found to retain the ability to accumulate phosphate.

Conditions Influencing Accumulation

Strain 55701 does not accumulate phosphate when it is allowed to grow at 25°C. but does so if the culture is kept at 35°C. for about 20 hrs. after growth has taken place at 25°C. Accumulation by the lysineless, pyrimidineless, and nicotinicless strain is dependent upon the concentration of the required growth factor. Accumulation by the nicotinicless strain is dependent also upon the nature of the growth-promotion substance supplied. For this mutant indole, tryptophan, kynurenine or hydroxyanthranilic acid will replace nicotinic acid (2, 5, 15) but very little accumulation has been observed when hydroxyanthranilic acid or nicotinamide was supplied. These facts are summarized in Table II.

TABLE II
*Effect of Concentration^a and Nature of Growth
Factor on Accumulation*

Strain number	Supplement mg./20 ml.		Acid-labile PO ₄ mg./g. wet wt.
4545	L(+)-Lysine monohydrochloride	1.0	5.5
4545	L(+)-Lysine monohydrochloride	3.0	3.7
37301	Cytidine sulfate	0.25	6.8
37301	Cytidine sulfate	1.0	3.7
65001	Indole	0.025	15.2
65001	Indole	0.15	2.5
65001	L-Tryptophan	0.04	15.0
65001	L-Tryptophan	0.2	5.8
65001	L-Kynurenine	0.04	13.2
65001	L-Kynurenine	0.2	2.0
65001	Hydroxyanthranilic acid	0.015	3.4
65001	Hydroxyanthranilic acid	0.08	1.6
65001	Nicotinamide	0.010	2.6
65001	Nicotinamide	0.050	1.7

^a The higher concentrations, in each case, are sufficient for optimal growth.

Fractionation of Phosphate in Extracts

By fractionation of trichloroacetic acid extracts from the 5 mutants, it was found that the greater part of the accumulation appeared, in each case, in the same fraction.

The extracts, at 0°C., were brought to about pH 4 with NaOH and chilled for 15 mins. after the addition of excess barium acetate. The resulting precipitate was removed by centrifuging and washed with 0.1 *N* HCl. The fraction in which most of the accumulation was found remained undissolved after repeated washing with 0.1 *N* HCl. Table III gives values for phosphate found in this fraction from a wild-type strain and from the 5 mutants.

TABLE III

Phosphate in Fraction Containing Accumulation

Strain number	Total acid-labile PO ₄ mg./g. wet wt.	PO ₄ in fraction A mg./g. wet wt.
Abbott 4A	1.7	0.9
4545	6.8	4.7
37301	5.0	3.6
39401	12.5	9.2
55701	3.8	3.0
65001	15.5	8.9

Isolation

This material, after being washed several times with 0.1 *N* HCl and then with water, was nearly pure. Further purification was obtained by dissolving it in ice-cold 2 *N* HCl, reprecipitating by the addition of barium acetate, and washing 5 or 6 times with water. When dried to constant weight over P₂O₅ at 25°C. material isolated from strain 55701 gave the following analysis:

	% Ba	% P
Calculated for Ba ₃ (PO ₃) ₆ · 1 H ₂ O	43.5	19.7
Observed	43.4	19.6

Fusion of the dried, amorphous barium salt in a micro carbon-hydrogen apparatus yielded analyses corresponding to 1.04% hydrogen and 0.53% carbon. It is evident that this material contains a small amount of organic impurity and, on fusion, gives up approximately one molecule of water/6 atoms of phosphorus. Whether the water is absorbed or chemically bound has not been determined. Analysis of the fused material gave the following results:

	% Ba	% P
Calculated for Ba(PO ₃) ₂	46.46	21.0
Observed	45.71	21.1

Since the higher polyphosphates do not form crystalline derivatives, the exact chemical nature of the isolated material remains to be determined. The analytical data suggest either hexapoly- or hexameta-phosphate.

Comparison with Poly- and Metaphosphates

Barium salts were prepared from a number of inorganic phosphates and the solubility of these in 0.1 *N* HCl compared with that of the isolated material. The compounds were the following: sodium pyrophosphate, Baker's; sodium dimetaphosphate

prepared by the method of Travers and Chu (17); sodium trimetaphosphate, prepared by the means of Beans and Kiehl (3); metaphosphoric acid, Baker's; and samples of tripolyphosphate, metaphosphate and septametaphosphate obtained from the Monsanto Chemical Co. All but the last two gave barium salts which were readily soluble in 0.1 *N* HCl. The metaphosphate and septametaphosphate samples appeared to be mixtures, part of which formed barium salts which were insoluble in 0.1 *N* HCl and, indeed, the metaphosphate was described as a mixture containing 15% tetrasodium pyrophosphate and 85% sodium metaphosphate. The composition of the septametaphosphate sample was not given. Barium salts obtained from these were soluble in cold 2 *N* HCl and reprecipitated by barium acetate.

Enzymatic Hydrolysis

Preliminary experiments have shown that wild-type strain Abbott 4 and mutant strain 55701 contain enzymes which hydrolyze the isolated compound as well as pyro-, dimeta- and trimetaphosphate to orthophosphate. The most active preparations were obtained by extracting dried, powdered mycelium with water. With the isolated material as substrate, comparison of the activities at 35 C. of enzyme preparations from wild-type and from mutant 55701 showed no difference which could account for accumulation by the mutant at this temperature.

Accumulation by Double Mutants

An interesting observation has been made on double mutants involving strains which accumulate phosphate. The double mutant of 37301 (pyrimidineless) and 38502, a pyrimidineless strain which accumulates large quantities of orotic acid (14), was found to accumulate neither orotic acid nor phosphate. Since it was considered possible that the mutation at the 38502 locus might prevent phosphate accumulation in any strain, the double mutant of 65001 (nicotinicless) and 38502 was obtained. In this case the presence of the pyrimidineless mutant gene did not affect the accumulation of phosphate.

Acid-Labile Phosphate not Extracted by Trichloroacetic Acid

A few experiments have indicated that in the strains which accumulate phosphate there are rather large quantities of phosphate which hydrolyze in 10 mins. but which are not extracted by trichloroacetic acid. This was found by Wiame (personal communication) to be the case in yeast. However, careful comparisons with wild-type have not been made and it is not known for certain whether accumulation of this material parallels accumulation of the soluble compound.

DISCUSSION

It may be concluded for several reasons that phosphate accumulation by the mutant strains described is not due to the presence of a second mutant gene common to all of them and unrelated to the mutation which gives rise to the additional growth requirement in each case.

First, the accumulation has not been observed in any of the wild-type strains from which the mutants were derived. Second, strains recovered from out-crosses of 3 of the 5 mutants have been found to retain the ability to accumulate phosphate. (Reisolated strains of the other two mutants have not been tested.) This result would be unlikely if a second gene were necessary, since the 3 genes are known not to be closely linked and segregation would, therefore, be expected to occur readily in at least 2 of the 3 cases. It would seem unlikely also that, in the double mutants described, the mutant form of gene 38502 would prevent accumulation by the pyrimidineless strain but not by the nicotinicless strain, if an unrelated gene were responsible for the accumulation.

In cases previously reported in *Neurospora* (4, 7, 8, 12, 14) accumulated metabolites have been looked upon as intermediates, or derivatives of intermediates, which would normally be transformed by the genetically blocked reaction. This appears to be ruled out as an explanation for the accumulation of the phosphate compound, since the behavior of the pyrimidineless double mutant 37301, 38502, would be difficult to understand on this basis. It has been assumed (13) that orotic acid accumulation by strain 38502 is prevented by the presence of the mutant form of gene 37301 because the 37301 reaction precedes the reaction involved in the 38502 mutation. Obviously then, the genetic block due to the mutation at the 38502 locus cannot prevent the accumulation of phosphate by strain 37301 for the same reason. It seems more probable that this accumulation results from the abnormal functioning of a general phosphorylating agent, the abnormality being due in turn to the genetically blocked reaction. This view is consistent with the fact that the same compound is accumulated by 4 strains involving at least 3, and very probably 4, different series of reactions.

The possibility that the accumulation results from an imbalance brought about by the necessity of supplying the required growth factor in the culture medium is suggested by the following observations: the dependence of the amount of accumulation upon the concentration, and, in one case, the nature of the growth factor supplied, and prevention of the accumulation by the introduction of a second genetic block further along in the reaction series. The fact that the amount of accumulation increases as the concentration of growth-promoting substance is decreased is difficult to interpret on the basis of the available facts. It is clear that, in the case of the nicotinicless strain, this is not due simply to limited growth, since very little accumulation was observed when

growth was limited with low concentrations of nicotinamide or hydroxyanthranilic acid. It seems probable, however, that limited growth is one of the essentials, since, in each case, greater accumulation was observed under this condition. More detailed experiments involving variation of both the length of the growth period and the concentration of growth factor may serve to clarify this point.

Little information concerning the structure of the phosphate compound is as yet available. The evidence presented is sufficient to make it certain that the compound is a poly- or metaphosphate with more than 3 atoms of phosphorus.

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Several samples of polyphosphates and metaphosphates were obtained through the kindness of Dr. R. S. Weatherly of the Monsanto Chemical Co., St. Louis, Mo.

SUMMARY

1. An inorganic, acid-labile phosphate compound has been found to be a normal constituent of *Neurospora*.

2. Four genetically and physiologically different mutants of *Neurospora* accumulate this compound. The accumulation has been shown to be dependent upon certain culture conditions and upon genetic constitution.

3. The chemical nature of the compound is not known but it appears to be a poly- or metaphosphate similar to, and possibly identical with, the metaphosphate found in yeast and in *A. niger* by other workers.

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Availability of Tryptophan from Various Products for Growth of Chicks

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INTRODUCTION

Recent studies on the amount of tryptophan required by the chick, and on the development of improved microbiological methods for the determination of tryptophan in crude materials, have facilitated investigations on determining the ability of the chick to utilize tryptophan from various products. In the present work the availability of tryptophan from raw, expeller and solvent-extracted soybean meals, fish meal, and casein was studied. The actual percentage of the dietary tryptophan from these products that was utilized to support growth of chicks was determined by comparing the growth rates obtained with the test material and with L-tryptophan. Further, other tests were made to determine the supplementary value of tryptophan with rations calculated to supply the minimal requirement of tryptophan contributed by the different products.

EXPERIMENTAL AND RESULTS

Care of Chicks

The procedure used was similar to that used in earlier work (1,2). Unsexed, day-old New Hampshire X White Leghorn chicks were used in these experiments. They were fed a commercial type starter mash for 10 days and the chicks were weighed on the first, fifth, and tenth days. The chicks that deviated the greatest from the mean performance were discarded. The birds were arrayed in the various experimental groups on the basis of their final weight and gain in weight. Ten birds were thus selected for each group and were fed the experimental diets for a period of 14 days. Weighings were taken at the end of the fifth, tenth, and fourteenth day.

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Composition of Rations

The basal diet used was a purified ration in which the majority of the protein was supplied by oxidized casein (3), gelatin, cystine and methionine and designed to be low in tryptophan, but adequate in other nutrients. The basal ration used in the first experiments was composed of the following: (in per cent) oxidized casein 12, crude casein 2, gelatin 10, mineral mixture (4) 5, corn oil 5, vitamin A and D concentrate 0.75, fish solubles (dry basis) 2, L-cystine 0.4, DL-methionine 0.8 and corn starch to 100. Each 100 g. of ration also contained the following amounts of vitamins (in mg.): thiamine 0.6, riboflavin 0.66, pyridoxine 0.66, calcium pantothenate 2.2, nicotinic acid 5.0, inositol 100, choline 200, *p*-aminobenzoic acid 0.2, biotin 0.02, pteroylglutamic acid 0.2, 2-methyl-1, 4 naphthoquinone 2.0, and mixed tocopherols 10. The vitamins, tryptophan and other supplements were added to the ration at the expense of the starch.

In the first experiments graded amounts of L-tryptophan, ranging from none to 0.15%, were added to the basal ration and the growth rates compared to those obtained with groups fed two levels of raw soybean meal,² expeller processed soybean meal or fish meal (menhaden). The nitrogen and tryptophan contents of the products used are shown in Table I. Tryptophan was determined microbiologically by the use of *S. faecalis* R as the test organism (5). Tryptophan was liberated from the samples by alkaline hydrolysis in the presence of cysteine (6).

TABLE I
Analytical Data on the Products Used
(Values are uncorrected for moisture and ash)

Product	Nitrogen per cent	Tryptophan per cent
Oxidized casein	14.15	0.025
Crude casein	15.12	1.10
Fish meal	10.31	0.65
Raw soybean meal	8.45	0.68
Exp. processed soybean meal	8.27	0.69
Solvent processed soybean meal	7.28	0.75

The results obtained when L-tryptophan and when fish meal, raw or expeller processed soybean meal was fed as a source of tryptophan are shown in Table II. The tryptophan content of the rations was computed from the data presented in Table I and from the composition of the rations. At the conclusion of these tests, the rations containing the crude products were analyzed for tryptophan, and it can be seen from the results presented in Table III that good agreement was obtained with the calculated values.

² The raw, defatted soybean oil meal and the solvent-extracted soybean oil meal were generously supplied by Dr. J. W. Hayward, Archer-Daniels-Midland Co., Minneapolis, Minnesota. The latter sample represents the standard production of the toasted soybean oil meal.

TABLE II
Tryptophan Availability in Various Products

Supplements to basal ration	Total tryptophan content	Gain of chicks ^a	Tryptophan in sample from growth data	Availability of tryptophan in sample
	<i>per cent</i>	<i>g.</i>	<i>per cent</i>	<i>per cent</i>
None	0.03	-8.0		
0.08% L-Tryptophan	0.11	28.0		
0.11% L-Tryptophan	0.14	68.0		
0.15% L-Tryptophan	0.18	132.0		
10% Fish meal	0.096	0.6	0.024	37
13.75% Fish meal	0.119	5.6	0.037	42
10% Raw soybean meal	0.098	-3.0	0.014	21
13.75% Raw soybean meal	0.124	-1.8	0.018	19
10% Exp. processed soybean meal	0.100	6.5	0.040	58
13.75% Exp. processed soybean meal	0.125	20.3	0.068	71

^a Average gain in weight for 10 chicks in each group at the end of 14 days on experiment.

From an interpolation of the growth rates obtained when L-tryptophan was fed, the amount of tryptophan available to the chick for growth was calculated for each level of the products tested. From these figures and the amount of tryptophan determined by analysis, the per cent availability of tryptophan in these products was calculated (Table II). It can readily be seen that the tryptophan utilized by the chick in the raw product was very low (20%), somewhat higher in fish meal (40%), and still higher in the processed soybean meal (65%). Further good agreement was obtained, particularly for the raw soybean meal

TABLE III
Comparison of the Calculated Tryptophan Content of Rations With Values Obtained by Analysis

Ration description	Tryptophan content	
	Calculated <i>per cent</i>	Determined <i>per cent</i>
Basal + 10% fish meal	0.096	0.100
Basal + 13.75% fish meal	0.119	0.124
Basal + 10% raw soybean meal	0.098	0.106
Basal + 13.75% raw soybean meal	0.124	0.119
Basal + 10% Exp. processed soybean meal	0.100	0.115
Basal + 13.75% Exp. processed soybean meal	0.125	0.137

and fish meal, for the two levels tested. The same conclusions were drawn from the results obtained after the chicks had been on experiment for 5 and 10 days. After the termination of these experiments a marked growth response was observed when tryptophan was added to the diets containing raw or processed soybean meal and fish meal, thereby adding additional evidence that tryptophan availability was the limiting factor in the previous test period. It should be pointed out that the products tested are relied upon only as a source of tryptophan since the other amino acids, vitamins (including nicotinic acid), and other essential nutrients are presumably present in adequate quantity in the basal ration as evidenced by data obtained in earlier work (1, 2).

Another experiment was designed to extend these observations and to determine the effect of tryptophan supplementation when each of the products was included in the ration at a level to approximate the minimum requirement for tryptophan (0.18%) if the tryptophan is completely available. Corresponding groups were fed the same diets plus L-tryptophan added in excess of the requirement at a level of 0.2% in order to supply sufficient supplementary tryptophan to give the maximum response within the limits of the adequacy of the rations in other constituents. In this manner the relative difference due to tryptophan supplementation would, therefore, give an indication of the degree of availability of tryptophan in the various products tested. The same samples of raw and expeller processed soybean meal and fish meal were used in this experiment. In addition, samples of solvent-extracted meal and crude casein were included. The latter product was used since previous studies (2) indicated that the tryptophan in casein was completely available.

The composition of the basal ration was changed somewhat, in that the crude casein was omitted and the amount of oxidized casein was reduced from 12 to 3%, which resulted in a reduction in the tryptophan contributed by the basal ration to 0.005%. In this manner the products tested supplied essentially all of the tryptophan, approximately one-half of the total protein and also were relied upon to contribute amino acids in addition to tryptophan. The total protein in the ration approximated 25%; however, it was necessarily varied for each ration depending on the amount of tryptophan in the product. The same experimental technique for the care of the birds and allocation of groups was used. The results obtained in these experiments are shown in Table IV.

The comparative results obtained with and without tryptophan supplementation for the groups receiving the raw and expeller processed meal and fish meal are in accord with the degree of tryptophan availability observed in the first experiment. Thus, the addition of tryptophan to the ration containing raw soybean meal resulted in a 2-fold increase in growth, tryptophan added to fish meal resulted in doubling the rate of growth, while supplementation of the expeller

processed meal gave only a 20% increase in the rate of growth. The results obtained with the solvent extracted meal were similar to those obtained with the expeller processed product. The difference in the growth rates observed when the processed meals were supplemented with tryptophan as compared to those noted when no tryptophan was added was analyzed statistically by an analysis of variance and was shown to be highly significant ($p < .01$ in each case). It is concluded, therefore, from these studies that, although the availability of tryptophan in the processed soybean meals was high, it was not complete.

TABLE IV

Effect of Tryptophan Supplementation on the Performance of Chicks Receiving Adequate Tryptophan from Various Products

Supplements to basal ration	Tryptophan added	Total tryptophan content	Average gain of chicks
	<i>per cent</i>	<i>per cent</i>	<i>g.</i>
27.8% Fish meal	None	0.185	73
27.8% Fish meal	0.2	0.385	141
26.5% Raw soybean meal	None	0.185	37
26.5% Raw soybean meal	0.2	0.385	97
26.1% Exp. processed soybean meal	None	0.185	129
26.1% Exp. processed soybean meal	0.2	0.385	155
24.0% Solvent processed soybean meal	None	0.185	128
24.0% Solvent processed soybean meal	0.2	0.385	153
16.4% Crude casein	None	0.185	107
16.4% Crude casein	0.2	0.385	123

The increase in the growth rate observed when casein was supplemented with tryptophan was not statistically significant and the difference in the mean gain indicated in the table may be attributed to two chicks in the unsupplemented group that gained only 49 and 56 g. during the experimental period. These results confirm earlier findings that the tryptophan in casein is essentially all available to the chick to support growth. Feed efficiency data obtained for these experiments were in accord with the growth data. The somewhat slower rate of gain observed with the group receiving raw soybean meal supplemented with tryptophan as compared to the results obtained with the processed meals may be due in part to the limiting availability of amino acids other than tryptophan in the raw product, since the protein supplements were relied upon to contribute a considerable portion of the

protein in the ration. This does not negate the validity of the findings since tryptophan was the most limiting nutrient. However, it does not permit an evaluation of the relative value of the different soybean meals for the amino acids other than those known to be relatively unavailable, namely, tryptophan, and methionine plus cystine. The somewhat slower rate of growth obtained with the casein diets cannot be readily explained. It may be possible that the processed meals supplied some amino acids that were slightly limiting in casein. Further tests are needed to determine the significance of this observation.

DISCUSSION

With the techniques used, not only is it necessary for the amino acids to be liberated and absorbed, and not be excreted in the urine, but it is also necessary that they be available to the animal simultaneously for protein synthesis when growth is used as a measurement as evidenced by several recent significant reports (7-9). With only one amino acid limiting the rate of growth, the availability can be expressed quantitatively in terms of that amino acid.

It is recognized that, when the crude supplements were added to the ration as a source of tryptophan, the crude protein content was increased by 5-8%. The basal ration contained approximately 24% crude protein while the rations containing the test products contained 29-32% protein. Whether this increase in protein content would affect the tryptophan requirement cannot be stated at present. At any rate, the protein levels in the test rations were comparable, and it is clear that the relative utilization of tryptophan from the different products is in accord with the percentage availability which was calculated from the data obtained with L-tryptophan supplements. Further experiments with higher test levels of the protein supplements would aid in clarifying the effect of the total protein ingested on the percentage availability of tryptophan.

The present work shows that, in addition to the sulfur amino acids, the availability of tryptophan is low in raw soybean meal and is increased when the soybean meal is processed. Studies were not carried out on overheated products; however, it seems quite likely that tryptophan may also be less available in meals treated in this manner (10, 11). From data obtained by other workers, it is quite possible that the low availability of tryptophan in the raw product would account in part for

the lower growth rates obtained with the raw product. In one of these studies (12) 48% raw soybean oil meal was fed as the sole source of protein, and, if the tryptophan content and availability were similar to those observed in the present work, the calculated amount of tryptophan available to the chick would be 0.1% or less, which is considerably below the amount required for optimum growth. It is clear, therefore, that not only are lysine, tryptophan and the sulfur amino acids the most limiting amino acids in commonly used food materials, but they may also be available to the animal to a limited extent. Whether the other amino acids, which occur more abundantly, at least in comparison to the amount required by the animal, are also incompletely available, and whether the availability is also greatly affected by the type of processing, remains to be determined.

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SUMMARY

The availability of tryptophan in various products has been determined, using the growth rate of chicks as the criterion of availability, and the results have been expressed quantitatively. It was found that the availability of tryptophan in raw soybean meal is low (20%), somewhat higher in fish meal (40%), and still higher in processed soybean meal (65%). This degree of availability was confirmed in subsequent tests in which the products tested were added at a level to supply the minimal requirement of the chick for tryptophan and the growth performance noted with and without additional supplements of L-tryptophan. The results show, therefore, that the availability of tryptophan for the growth of chicks is limited and is affected by the processing of soybean meals. The tryptophan in casein was shown to be essentially all available.

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Studies in the Respiration of the White Potato. II

Terminal Oxidase System of Potato Tuber Respiration¹

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INTRODUCTION

In a previous paper from this laboratory (10), evidence was presented which cast doubt on the validity of the use of catechol in identifying the terminal oxidase of potato respiration as tyrosinase. Catechol "apparently acts primarily as a cell poison, capable of disrupting completely the normal metabolic processes on which viability of the cells depends, and only secondarily as a substrate for tyrosinase." As a result of this study, a reinvestigation of the nature of the oxygen transporting enzyme system of potato respiration was undertaken.

The results to be reported below make improbable the popularly held belief that catechol oxidase (tyrosinase) functions as the terminal oxidase in potato respiration. Rather, two other enzymes appear to fill this role.

EXPERIMENTAL

Materials and Methods

White potatoes were bought in neighborhood stores when needed. Cylinders of potato tissue, 7.7 mm. in diameter, were cut with a cork borer. Slices 0.5 mm. thick were prepared with a hand microtome and razor. To remove debris from the cut surface of cells, the slices were placed in a flask, and washed with a stream of tap

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water, at about 10–15°C. With the exception of the data of Table V, the results reported here are on slices washed 16–22 hours.²

Homogenates were prepared, just before use, with glass homogenizers (9). The tubes were kept in ice water during homogenization, and until the samples were used. Homogenates were heat-denatured by placing them, in tubes, in a bath of boiling water for 15 min.

Carbon monoxide was generated by dropping reagent grade formic acid into hot reagent grade sulfuric acid. The gas was then washed through 10% sodium hydroxide and through concentrated sulfuric acid, mixed with the indicated amount of U.S.P. oxygen, and stored over water for 0.5–1 hr. before use. Preliminary experiments with carbon monoxide and potato respiration revealed the absorption of gas in Warburg respirometers containing a mixture of 95% CO + 5% O₂ and KOH-wet filter paper, in the absence of any tissue. The amount of gas taken up varied with the temperature and with the area of the paper. Under our conditions, in 17 cc. Warburg vessels, the gas uptake at 31°C was ca. 12 μ l/hr., and at 25°C. was ca. 8 μ l/hr. The filter paper used was Whatman Number 40 of approximately constant area (5 cm²). A correction of 10 μ l/hr. was found to be applicable at 31°C. when the gas mixture was 80% CO–20% O₂. All the data reported here were corrected accordingly.

Cytochrome c was prepared by the method of Keilin and Hartree (4) and standardized spectrophotometrically.

Warburg respirometer vessels were made opaque by painting regular vessels with Glyptal³ paint mixed with carbon black.

For the studies on photoreversibility of carbon monoxide inhibition, the light source was either a General Electric Number 3 mercury vapor lamp, or a Mazda photoflood Number 1 lamp, fitted for underwater use. During use, the mercury vapor or the tungsten filament was about 2 in. directly below the bottoms of the Warburg vessels. Incident light intensities were measured approximately with a General Electric photographic exposure meter. The reading, in foot candles, was converted to g-cal./min./cm.² by multiplying by 2.14×10^{-5} .

All results reported in this paper represent values obtained from at least duplicate determinations.

RESULTS

I. STUDIES ON INTACT POTATO SLICES

Inhibition of Respiration by Carbon Monoxide

Warburg and others (6,8,14) have shown that cytochrome oxidase is inhibited by carbon monoxide in the dark, but that strong illumination reverses this inhibition. The effectiveness of light for the reversal of the carbon monoxide inhibition is dependent on its wave length. Maximum reversal takes place at $\lambda = 450 \text{ m}\mu$ (8). As a convenient approximation we have used the 436 m μ line of the mercury spectrum, isolated by placing Corning glass filters Number 5113 and Number 3389 over the General Electric H-3 lamp.

² It became apparent after work had been under way, that certain aspects of the respiration of the potato changed qualitatively with increasing periods of washing of the slices. The nature of the change will be studied further and reported later.

³ General Electric Corp., Schenectady, N. Y.

To observe the response of potato respiration to carbon monoxide in the dark and in the light, 25 potato slices in 3.0 cc. of $M/100$ phosphate buffer, pH 6.1, were exposed to 95% CO + 5% O₂ in opaque Warburg vessels and in clear vessels illuminated with strong light of $\lambda = 436 \text{ m}\mu$. The respiration of control slices in 95% N₂-5% O₂ was determined simultaneously. Table I shows the results of some representative experi-

TABLE I

Effect of 95% CO-5% O₂, in Dark, and in Strong Light of $\lambda = 436 \text{ m}\mu$, on the Respiration of Potato Slices

Temperature of measurement	95% N ₂ -5% O ₂ resp./hr.	CO dark		CO light	
		Resp./hr.	Per cent inhib.	Resp./hr.	Per cent inhib.
$^{\circ}\text{C}$	μl	μl		μl	
25	27	17	37	27	0
31	50	35	30	48	4
31	45	32	29	42	7
31	58	38	35	52	10
31	64	39	39	62	3

ments. In a total of some 30 experiments 95% carbon monoxide in the dark effected inhibition of respiration over a range of 10-65%, with a majority falling between 25-45%. In all cases, this inhibition was completely or nearly completely reversed by light.

The participation of cytochrome oxidase in potato respiration is strongly indicated by these results.

The function of the light in the above experiments is to effect the photodissociation of the carbon monoxide-cytochrome oxidase complex. Therefore, turning off the light should cause the carbon monoxide inhibition to become manifest. Table II shows the results of 3 experiments to determine whether or not this was the case in potatoes. In these experiments the CO inhibition set in on turning off the light. However, of a total of 12 experiments, there were 6 experiments in which turning off the light did not result in the appearance of CO inhibition. In these 12 experiments all light was excluded from the bath after the extinction of the mercury lamp.

Further, if potato slices were irradiated in strong light of $\lambda = 436 \text{ m}\mu$ in the presence of air it was found that in 7 out of 16 experiments the respiration of these slices was no longer inhibited by carbon monoxide. The respiration of non-irradiated control slices was inhibited some 25-45%. We have no satisfactory explanation for these phenomena.

Since the photochemical spectrum of CO-cytochrome oxidase shows that there is only negligible reversal of the CO inhibition of cytochrome oxidase activity by red light ($\lambda > 620 \text{ m}\mu$) as contrasted with blue ($\lambda \leq 450 \text{ m}\mu$), the effect of red and blue light on the CO inhibition of potato respiration was determined and compared.

Two Mazda photoflood Number 1 bulbs were fitted for underwater use. The light from one was passed through Corning filters Number 5113 and Number 3389 as above,

TABLE II
Reversibility of the Effect of Light on CO Inhibition of Potato Respiration

		Per cent inhibition		Per cent reversal of inhib. in clear vessels
		CO dark (painted vessels)	CO light (clear vessels)	
1	Light on ^a	42	11	74
	off	34	38	0
	on	22	0	100
2	Light on	33	0	100
	off	58	25	57
	on	44	11	75
3	off	56	45	20
	Light on	50	0	100
	off	43	43	0

^a Each observation period was of 1 hr. duration.

to give blue light of $\lambda = 430-470$ m μ . The light from the other was passed through Corning filters Number 978 $\frac{1}{2}$ St and Number 2403, giving red light of $\lambda = 620-670$ m μ . Each lamp was connected to a separate Variac transformer so that the light intensities could be varied independently. Approximate measurement of the energies of the lights were obtained with the use of a General Electric photoelectric exposure meter. Suitable corrections were made for the lower sensitivity of the meter to the red light.

TABLE III
*Comparison of the Effect of Red and Blue Light on CO Inhibition
of Potato Respiration*

The energy of the light was $1.2-1.3 \times 10^{-3}$ g. cal./min./cm.²

Exper.		95% N ₂ - 5% O ₂	95% CO-5% O ₂					
			Dark		Red light		Blue light	
			O ₂ ^a	Per cent inhib. ^b	O ₂ ^a	Per cent inhib. ^b	O ₂ ^a	Per cent inhib. ^b
1	1st hr.	30	10	67	16	47	27	17
	2nd and 3rd hr.	53	35	34	34	36	52	2
2		58	46	21	48	17	57	2
3		52	35	33	34	35	38	8

^a μ l. O₂ uptake/hr./25 slices, in 3 cc. *M*/100 phosphate buffer, pH 7.0.

^b Compared to respiration in 95% N₂-5% O₂.

The intensities of the red and of the blue lights were adjusted so that the energy entering the Warburg flasks was about 1.2×10^{-3} g.-cal./min./cm². This energy value was 50% higher than that which preliminary experiments indicated was needed with the blue light to effect reversal of the inhibition of respiration by CO.

The results of 3 experiments are given in Table III. It can be seen that, even though the energy of the incident red light was equal to that of the blue light, the red light did not reverse the carbon monoxide inhibition. This fact thus presents additional evidence that cytochrome oxidase participates in the respiration of the potato.

Kubowitz (7) has reported that tyrosinase is inhibited by carbon monoxide and that light has no effect on this inhibition. We have confirmed these results. Tyrosinase⁴ was inhibited *ca.* 80–85% by 95% CO, and *ca.* 70% by 80% CO. Light did not reverse either inhibition. Potato respiration, however, was usually unaffected by 80% CO. Whenever an inhibition was observed, it was less than 20% and was reversed by light. In view of these results, together with those given in Table I for 95% CO, the participation of tyrosinase in potato respiration would seem most improbable.

*Effect of Variation of the Partial Pressure of O₂ on
Potato Respiration*

The cytochrome oxidase system has been reported (2,16) as exhibiting full activity where the *p*O₂ in the gas phases was as low as 0.24–2.5 mm. Hg. Accordingly, the effect of lowering the *p*O₂ in the gas phase on the respiration of potato slices was studied. Gas mixtures

TABLE IV
*Effect on Potato Respiration of Varying *p*O₂*

<i>p</i> O ₂ , mm. Hg.	19	38	76
Per cent inhibition compared to air }	38	9	0
	34	21	5
	40	14	

containing 20, 10, 5, and 2.5% O₂ with 80, 90, 95, and 97.5% N₂, were prepared by mixing suitable quantities of pure gases (Ohio Chemical Company) or were purchased as prepared mixtures. Typical results are given in Table IV. The fact that the respiration of potato slices is lowered in gas mixtures where the pressure of O₂ is either 19 mm. Hg.

⁴ Kindly supplied by Drs. Nelson and Dawson of Columbia University.

(2.5%) or 38 mm. Hg. (5%) suggests: (1) the rate of diffusion of O_2 from the gas phase into the liquid phase is limiting; (2) the diffusion coefficient is so low that the pO_2 inside the slice is very low; (3) some enzyme in addition to cytochrome oxidase is functioning in the slice respiration.

The first possibility was eliminated by varying the shaking speed of the Warburg vessel from 60 to 120 strokes/min. (Dixon). The respiratory rate rose until the shaking speed was 100 strokes/min, after which no further increase took place.

The second possibility, namely, that the rate of diffusion of oxygen from the liquid into the slice is too low, is thought to be unlikely because of the following considerations: (1) the respiration of slices 0.3 mm., or 1-2 cells, thick showed a sensitivity to lowered oxygen pressure similar to that shown by the usual 0.5 mm. slices, which were 3-4 cells thick; (2) further, since both layers of cells of a 2 cell thick tissue are exposed to the outside source of oxygen, the rate of diffusion of oxygen into the tissue would not be expected to be a limiting factor when the tissue has a Q_{O_2} of 1 or less; (3) the oxygen pressure at the center of the slice has been calculated to be sufficient for maximum cytochrome oxidase activity.

For the determination of oxygen pressure at the center of a tissue slice Warburg (11) derived the following equation:

$$U = C - \left(\frac{\alpha}{2D} \right) \left(\frac{H^2}{4} \right),$$

where $U = pO_2$ in atm. at center of tissue;

$\alpha = \text{ml. } O_2/\text{min.}/\text{ml. of tissue (approx. } 2 \times 10^{-3} \text{ in 5\% } O_2\text{);}$

$H = \text{thickness of tissue in cm. (} 3 \times 10^{-2}\text{); and}$

$D = \text{diffusion constant.}$

Krogh (5) gives a figure of 1.4×10^{-5} ml. $O_2/\text{cm.}^2/\text{min.}$ where the pressure gradient is 1 atm./cm. Since a potato slice 0.3 mm. thick is only 2 cells thick, it is most unlikely that D for potato tuber is less than that for muscle. We shall assume Krogh's value for D .

$C = \text{external pressure in atm. For 5\% } O_2, \text{ this is 0.05 atm.}$

Calculation leads to a value of

$$U = 0.035 \text{ atm. (26 mm. Hg.).}$$

This partial pressure of oxygen is significantly higher than that usually given for the pressure required for maximum cytochrome oxidase action (0.5-2.5 mm. Hg.).

These considerations show that neither the rate of diffusion of oxygen into the slice, nor the partial pressure of oxygen inside the slice would

account for the observed decrease in respiration in 5% oxygen if all the respiration were mediated by cytochrome oxidase.

The third possibility, namely, that an enzyme in addition to cytochrome oxidase is functioning as a terminal oxidase in potato slice respiration, must be seriously entertained. The sensitivity of this enzyme is apparently such that its reduced activity accounts for the lowered respiration rate of potato slices in gas mixtures where the O_2 pressure is 19 or 38 mm. Hg.

The ensuing section will present independent evidence for the presence in potato homogenates of cytochrome oxidase, as well as another enzyme requiring a relatively high pO_2 for maximum action.

II. STUDIES ON POTATO HOMOGENATES

Evidence for Cytochrome Oxidase

The manometric assay of tissue homogenates for cytochrome oxidase depends on the fact that, in the preparation of the homogenate, the concentration of cytochrome c is reduced to a level such that the cytochrome system functions suboptimally, or not at all. The addition of excess cytochrome c then causes an increase in the oxidation of substrates such as *p*-phenylenediamine (ppda) or ascorbate, which increment is taken as a measure of the cytochrome oxidase activity of the tissue.

TABLE V
Activity of Homogenate + Cytochrome c on ppda
 $\mu l O_2$ uptake/30 min.

Homogenate	Homogenate + cytochrome c	Increment (cytochrome oxidase)
13	22	9
18	27	9
20	37	17
12	25	13
10	44	34

Each vessel had 1.0 cc. of homogenate (6 g. potato slices, 9 cc. H_2O)^a 1 cc. of *M*/10 phosphate buffer, pH 7.4, and 0.3 cc. of water or of 3.86×10^{-4} *M* cytochrome c. The side arm had 0.5 cc. (5 mg.) of ppda in water, pH 6. The midwell contained 0.3 cc. of 20% KOH. Corrections for autoxidation of ppda were made.

^a The concentrations of homogenates are much higher than those ordinarily used with animal tissues, and the cytochrome oxidase values are quite low. It must be remembered, however, that potatoes have less than 2% protein, as compared to about 20% for liver, and that the respiratory QO_2 is quite low.

A. *Action of Homogenate and Cytochrome c on ppda.* The cytochrome oxidase activity of potato homogenates at pH 7.4 was determined using ppda as a substrate. Table V gives the results of 5 typical experiments.

The data show that the addition of cytochrome c to homogenates caused variable but definite increases in the oxidation of ppda. At pH 6.1, the action of the homogenate alone on ppda, and the oxidation increment caused by cytochrome addition was either equal to, or, more often, slightly lower than, the action at pH 7.4.

Since the possibility exists that the action of cytochrome c might be referable to some indirect oxidation not related to cytochrome oxidase activity, we investigated the cytochrome c increment with respect to two known characteristics of cytochrome oxidase: (1) a relative insensitivity to lowered pO_2 ; (2) a photo-reversible inhibition by mixtures of carbon monoxide and oxygen.

1. Cytochrome oxidase activity in a gas mixture of 97.5% N_2 -2.5% O_2 ($pO_2 = 19$ mm. Hg.) was determined and compared with the activity of the same homogenates in 20% O_2 -80% N_2 . The results are given in Table VI.

TABLE VI
Effect of Lowered pO_2 on Potato Cytochrome Oxidase Action on ppda
 μl O_2 uptake/30 min.

In 20% O_2 -80% N_2			In 2.5% O_2 -97.5% N_2		
Homog.	Homog. + cyt. c	Cyt. oxid.	Homog.	Homog. + cyt. c	Cyt. oxid.
13	22	9	3	15	12
18	27	9	6	18	12
20	37	17	5	25	20

Experimental setup as given in Table V.

It can be seen that the increment in O_2 uptake caused by adding cytochrome c to potato homogenate in the presence of ppda is not decreased, but, if anything, is slightly increased by lower pO_2 's. This curious fact was observed in many similar experiments.

2. The effect on cytochrome oxidase action on ppda, of gas mixtures of 95% CO -5% O_2 , in the dark and in the presence of strong light of $\lambda = 436 m\mu$ was determined. The results are given in Table VII.

It will be seen that the CO inhibition is markedly reversed by light of $\lambda = 436 m\mu$.

TABLE VII
Effect of 95% CO + 5% O₂ on the Increment of Oxygen Uptake Due to Potato Cytochrome Oxidase Action on ppda

95% N ₂ + 5% O ₂	95% CO + 5% O ₂			
Cyt. ox.	Dark		Light	
	Cyt. ox.	Inhib. ^a	Cyt. ox.	Inhib. ^a
μl	μl	per cent	μl	per cent
28	2	93	21	25
37	10	73	29	22

Each vessel contained 1 cc. of homogenate (6 g. potato slices, 12 cc. H₂O), 1 cc. of *M*/10 phosphate buffer, pH 7.0, and 0.4 cc. of water or 4×10^{-4} *M* cytochrome c in the main room. The side arm had 0.4 cc. (4 mg.) of ppda in water, pH 6.0. The midwell contained 0.3 cc. of 20% KOH.

^a Compared with 95% N₂ + 5% O₂.

The increment of ppda oxidation found following addition of cytochrome c to potato homogenates, the insensitivity of this increment to lowered partial pressures of oxygen, and the photoreversible inhibition by carbon monoxide of the realization of this increment can be taken as strong evidence for the presence in homogenates of a cytochrome oxidase comparable to animal and yeast cytochrome oxidases.

TABLE VIII
Potato Cytochrome Oxidase Action on Ascorbate
 μl O₂ uptake/40 min.

Gas phase—Air			
pH 6.1		pH 7.0	
Homog.	Homog. + cyt. c	Homog.	Homog. + cyt. c
28	90	26	126
48	66	51	72
35	47	102	141

Each vessel contained 1 cc. of homogenate (6 g. potato slices, 8 cc. H₂O), 1 cc. of *M*/10 phosphate buffer, pH 6.1 or pH 7.0, and 0.4 cc. of water or of 2×10^{-4} *M* cytochrome c in the main room. The side arm had 0.4 cc. (4 mg.) of ascorbate in water, pH 6.0. The midwell contained 0.3 cc. of 20% KOH. Corrections were made for autoxidation of ascorbate in the presence of boiled homogenate.

B. Action of Homogenate and Cytochrome c on Ascorbate. The action of potato homogenate on ascorbate, with or without added cytochrome c at pH 6.1 and at pH 7.0 was greater than its action on ppda. Table VIII gives some typical results at these pH's with 6 different preparations.

As can be seen, the addition of cytochrome c caused a variable but significant increment in the oxidation of ascorbate.

Lowering of the partial pressure of oxygen from 152 mm. to 38 mm. Hg. was without effect on the increased oxidation of ascorbate resulting from cytochrome c addition to the homogenates. The action of potato cytochrome oxidase on ascorbate was inhibited about 80% by 95% CO + 5% O₂ in the dark, and this inhibition was decreased to about 40% by strong light of $\lambda = 436 \text{ m}\mu$. With ascorbate, therefore, as with ppda, potato homogenates show typical cytochrome oxidase behavior.

*Evidence for an Enzyme Sensitive to Lowered pO₂
but Insensitive to CO*

A. Homogenate Action on ppda. The action of homogenates on ppda without added cytochrome c is markedly sensitive to lowered partial pressures of oxygen. Table VI provides data for the comparison of the action of homogenates on ppda in air with that in 2.5% O₂-97.5% N₂. Table IX summarizes similar data for homogenate action in air vs. 5%

TABLE IX
Effect of Lowered pO₂ and of CO on Potato Homogenate Action on ppda

pH	Air	95% N ₂ -5% O ₂		95% CO-5% O ₂			
	O ₂ ^c	O ₂ ^c	Inhib. ^a	Dark		Light	
				O ₂ ^c	Inhib. ^b	O ₂ ^c	Inhib. ^b
			<i>per cent</i>		<i>per cent</i>		<i>per cent</i>
7.0	17	6	65	6	0	8	0
7.0	20	9	55	10	0	10	0
6.1	12	3	75	3	0	3	0

Each vessel had 1 cc. homogenate (8 g. slices, 11 cc. H₂O), 1.0 cc. M/10 phosphate buffer of indicated pH plus 0.5 cc. H₂O in the main room. Side arm had 0.5 cc. ppda (5 mg.) H₂O, pH 6.0; midwell contained 0.2 cc. of 20% KOH.

^a Compared with air.

^b Compared with 95% N₂-5% O₂.

^c μl O₂ uptake/20 minutes.

O₂-95% N₂. From these two tables it can be seen that a lowering in the oxygen concentration from 20% to 5% results in a decrease of enzyme activity of 60-70%, while a lowering to 2.5% O₂ effects a reduction of 80-85%.

The insensitivity of homogenate action on ppda to carbon monoxide (95% CO + 5% O₂) is apparent from the data given in Table IX. The failure of carbon monoxide to inhibit the homogenate action on ppda shows that tyrosinase cannot be responsible, even indirectly,⁵ for this oxidation, since carbon monoxide is strongly inhibitive of this enzyme's activity.

That the homogenate action on ppda involves a heavy metal enzyme is suggested by the fact that both cyanide and azide are inhibitory. At pH 6.1, 5×10^{-3} M NaCN caused an inhibition of ca. 90% while 2×10^{-2} M NaN₃ resulted in ca. 50% inhibition.

B. Homogenate Action on Ascorbate. The action of potato homogenates on ascorbate was markedly decreased when the oxygen concentrations were lowered from 20% to 5%. Table X presents the data of 3

TABLE X
Effect of Lowered pO₂ on Homogenate Action on Ascorbate
μl O₂ uptake/hr.

Air	5% O ₂ -95% N ₂	Per cent decrease
101	49	51
78	32	59
63	32	49

Each vessel contained 1 cc. homogenate (10 g. slices, 14 cc. H₂O), 1 cc. *M*/10 PO₄, pH 6.1 in the main room. In the side arm was 4 mg. (0.4 cc.) ascorbate in H₂O, pH 6.0. The midwell contained 0.2 cc. of 20% KOH.

experiments. It is apparent that the oxidation of ascorbate by the homogenates was reduced 50-55% by the lowering of the pO₂.

The effect of carbon monoxide on ascorbate oxidation by homogenates differed from its effect on their ppda oxidation. The oxidation of ascorbate was inhibited 0-30% by CO in the dark. Where inhibition was present it was reversed by light of $\lambda = 436$ mμ.

DISCUSSION

The foregoing experiments demonstrate that there is involved in potato tuber respiration an enzyme system that is photoreversibly

⁵ Tyrosinase does not directly oxidize ppda as a substrate.

inhibited by CO. The intensity of blue light needed to reverse the CO inhibition is of the same order of magnitude as that previously reported for animal cytochrome oxidase (8). The evidence thus points to the conclusion that cytochrome oxidase is functioning in potato respiration. If this is the case, it would appear likely that cytochrome c should be found in the potato. In spite of strenuous efforts, we have not succeeded in finding any cytochrome c. However, Keilin, in 1925, with the use of a micro spectroscope, reported seeing the bands of the cytochrome spectrum in potato tuber slices (3).

It is difficult to envision the functioning of tyrosinase in potato tuber respiration in view of the preceding data, namely, (1) tyrosinase action is inhibited some 85% by 95% CO-5% O₂ and about 70% by 80% CO-20% O₂, while tuber respiration is inhibited about 40% by 95% CO-5% O₂ and little, or not at all, by 80% CO-20% O₂; (2) the CO inhibition of tyrosinase is not affected by light.

Carbon monoxide exerts its inhibitory action on cytochrome oxidase because the carbon monoxide competes with oxygen for the enzyme molecule. If k_{O_2} and k_{CO} are the equilibrium constants respectively for the combination of O₂ and of CO with cytochrome oxidase, then one may express a relative affinity constant K as $\frac{K_{O_2}}{K_{CO}}$.

It has been shown by Warburg (13) that, as a first approximation,

$$\frac{n}{1-n} \cdot \frac{(CO)}{(O_2)} = K,$$

where the respiration in oxygen is 1, the respiration in the given mixture of carbon monoxide and oxygen $\frac{(CO)}{(O_2)}$ is n , and the amount of inhibited respiration is $1-n$.

K for yeast is about 9-10 (12,15). If we assume a K of 9 for potato, and further assume that all the respiration goes through cytochrome oxidase, then for 95% CO-5% O₂

$$\frac{n}{1-n} \cdot 19 = 9,$$

$$n = 0.32.$$

Therefore the inhibition caused by 95% CO should be about 70%. Actually the inhibition found with 18-22 hr. washed potato slices was about 35-40%.

One possible explanation for this smaller than expected inhibition is that K for potato cytochrome oxidase might be about 28-29. Another explanation is that cytochrome oxidase is mediating only about 55% of the respiration in these slices, and that a CO-insensitive enzyme is

taking care of the rest. The anticipated 70% inhibition of the cytochrome oxidase system would then cause an inhibition of 38% of the total respiration.

The finding of a sensitivity of the respiration to lowered oxygen pressures can also be explained in two ways. The first explanation is that the pO_2 of oxygen inside the slice is so low that even cytochrome oxidase is inhibited. In the section dealing with the effect of lowered oxygen pressure in potato respiration, this possibility was ruled out. The second explanation is that there is an enzyme participating in the respiration whose maximum activity is not elicited at lower partial pressures of oxygen.

Therefore, the evidence obtained by the use of CO together with the evidence from experiments using varying pO_2 , attest to the presence in in potatoes of 2 terminal oxidases, one photoreversibly inhibited by CO and whose action is not reduced by lowered partial pressures of oxygen; the other whose action is markedly reduced by lowered pO_2 , and which is insensitive to CO.

The section dealing with potato homogenates supports the foregoing conclusions. The presence of cytochrome oxidase in potato tuber is demonstrated by the increased oxidation of ppda and ascorbate found on adding cytochrome c to potato homogenate. That this cytochrome oxidase is at least similar to animal and to yeast cytochrome oxidase is shown by the fact that CO photoreversibly diminishes this increment in oxidation approximately 70%. The increment is not diminished by lowering the pO_2 from 150 mm. Hg. to 38 mm. Hg.

The presence in potato homogenates of an enzyme insensitive to CO but sensitive to lowered pO_2 is shown by the fact that the action on ppda and ascorbate of homogenates without added cytochrome is markedly decreased by lowering the partial pressure of oxygen. The action on ppda, at least, is not sensitive to carbon monoxide. The oxidation of ascorbate by homogenate without added cytochrome would appear to involve residual cytochrome oxidase as well as the enzyme sensitive to lowered partial pressures of oxygen.

Whether the postulated respiratory system that is insensitive to CO but sensitive to lower pO_2 is identical with the comparable enzyme found in homogenates cannot be stated with assurance on the basis of the data presented here. The next paper will give evidence suggesting their identity, as well as some data on the changes found in the respiratory pattern of potato slices after washing for varying periods of time.

SUMMARY

1. The respiration of potato slices involves the participation of an enzyme system whose activity is photoreversibly inhibited by carbon monoxide and is unaffected by lowered partial pressure of oxygen. This enzyme system is similar to the yeast and animal cytochrome oxidase system.

2. In addition to cytochrome oxidase, potato respiration involves an enzyme that is carbon monoxide-insensitive, but which requires relatively high pO_2 for optimal activity.

3. Independent evidence for the presence of cytochrome oxidase in potatoes was obtained through the use of homogenates. Addition of beef cytochrome c to potato homogenates causes an increased oxidation of *p*-phenylenediamine and of ascorbate. The increment is photoreversibly inhibited by carbon monoxide and is not diminished by lowered partial pressures of oxygen.

4. Potato homogenates without added cytochrome c oxidize *p*-phenylenediamine and ascorbate. This oxidation requires a relatively high partial pressure of oxygen and is essentially unaffected by carbon monoxide.

5. The view that tyrosinase is the terminal oxidase of potato respiration is not supported by these data.

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The Enzymatic Liberation of Pantothenic Acid ¹

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INTRODUCTION

The higher level of pantothenic acid found in certain foods when tested by the chick, as contrasted to the microbiological assay, has been a subject of some discussion (2). Numerous reports (6, 8, 10, 11) show that pantothenic acid occurs in tissues in bound forms which are not available to the test organisms employed. Determination of the total pantothenic acid content of biological materials necessitates the use of hydrolytic enzymes, as the vitamin is readily destroyed by either acid or alkali. However, this technique generally does not insure maximum liberation.

Recently, the purified coenzyme for acetylation (4) was found by Lipmann and his associates (5) to contain pantothenic acid. The simultaneous action of a purified alkaline intestinal phosphatase and a pigeon liver enzyme liberated from the coenzyme preparation almost the theoretical amount of pantothenic acid as calculated from its β -alanine content. In the present work a series of natural products has been assayed for pantothenic acid under 3 different conditions: (a) without hydrolysis, (b) after hydrolysis with mylase P (1), and (c) after hydrolysis with the pigeon liver enzyme and alkaline phosphatase (7). The unpublished details of the latter technique were kindly given us by G. D. Novelli and F. Lipmann, Massachusetts General Hospital, Boston, Mass. The liver enzyme was also prepared from chicken liver and its activity compared with the pigeon liver enzyme.

EXPERIMENTAL

As fresh chicken liver is more readily available than pigeon liver it was decided to attempt preparation of the liver enzyme from the former source. This substitution has been stated to be a satisfactory procedure (G. D. Novelli, personal communication).

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However, an experiment was carried out to compare the hydrolytic qualities of the two preparations. Livers were taken from freshly killed chicken or pigeon, cooled, minced, and homogenized with 20 volumes of cold acetone in a Waring blender. The precipitate was washed with acetone and ether. The powder was extracted with 10 times its weight of ice cold 0.02 *M* sodium bicarbonate and centrifuged. The enzyme is labile at room temperature and should be kept frozen in small quantities. The preparation of acetone powder liver extract is described in detail in the paper by Kaplan and Lipmann (3). The alkaline phosphatase was prepared from calf intestine by the method of Schmidt and Thannhauser (9). The purified solution had an activity of 1150 phosphatase units/mg. of N, and a concentration of 1484 units/ml. This enzyme solution was also kept frozen in small portions and was found to be stable under these conditions for at least 4 months.

A boiled extract of chicken liver was chosen as the substrate on which to compare the activity of the two enzymes. The extract was prepared by mincing the liver of a freshly killed chicken into twice its weight of boiling water. The mixture was macerated in a mortar and filtered. An aliquot of the filtrate was assayed without enzyme treatment to determine the free pantothenic acid value. A series of tubes were set up

TABLE I
Comparison of Chicken and Pigeon Liver Enzyme Preparations for Liberating Pantothenate from a Boiled Extract of Chicken Liver^a

Tube no.	Substrate added	Increased pantothenate found after treatment with intestinal phosphatase plus	
		Chicken enzyme ^b	Pigeon enzyme ^c
	ml.	γ /ml.	γ /ml.
1	0.2	24.7	16.2
2	0.4	25.2	17.5
3	0.5	22.3	16.3
4	0.7	25.4	17.5

^a Free pantothenic acid content, 0.7 γ /ml.

^b Enzyme blank, 0.24 γ pantothenate per tube.

^c Enzyme blank, 0.22 γ pantothenate per tube.

containing from 0.0 (blank) to 0.7 ml. of boiled liver extract filtrate, 0.05 ml. of the chicken or pigeon liver enzyme, 0.2 ml. of the intestinal phosphatase, 0.05 ml. of 0.1 *M* sodium bicarbonate and distilled water to a final volume of 1.0 ml. The tubes were incubated for 4 hrs. at 37°C., 4.0 ml. of distilled water added, and assayed for pantothenate by the method of Neal and Strong (6). The results are given in Table I. It is apparent that the chicken liver enzyme preparation was somewhat more effective than that from the pigeon liver in liberating bound pantothenate. The boiled liver extract contained about 30 times as much bound as free pantothenate.

An indication that the liberated growth factor is actually pantothenic acid was obtained by destroying it with an alkali treatment. An extract

of chicken liver was prepared in the usual way with the exception that the time interval between the death of the animal and the preparation of the boiled extract of its liver was held to an absolute minimum. In this case almost 100-fold increase in pantothenic acid activity was obtained after enzymatic digestion. Autoclaving for 10 min. at 15 lbs. pressure in 0.11 *N* NaOH completely destroyed the activity of the digested extract. After neutralization, almost complete recovery of a quantity of added calcium pantothenate was found (Table II).

TABLE II
Alkali Destruction of Enzymatically Liberated Pantothenic Acid

	Pantothenate γ /ml.
1. Boiled chicken liver extract	0.38
2. Boiled chicken liver extract, digested	36.75
3. No. 2, autoclaved with NaOH	0.00
4. No. 3, plus 20 γ pantothenate/ml.	19.80

In a further experiment, a series of natural products was assayed for free, mylase P-liberated and liver enzyme-intestinal phosphatase-liberated pantothenic acid. The experimental conditions were identical with those used above, except that a Waring blender homogenate of the material to be assayed replaced the boiled liver extract. In each case it was desirable, in order to achieve maximum liberation, to use a minimum amount of substrate, thus keeping enzyme concentration in excess. For instance, it was found that when 200 mg. rather than 40 mg. of salmon was incubated with 0.05 ml. of the liver enzyme and 0.2 ml. of the intestinal phosphatase only 52.8% of the pantothenic acid was liberated. The materials studied here were used in amounts ranging from 1 to 40 mg. and, for each product, incubation mixtures were set up in duplicate. In a second experiment, the enzyme concentration was maintained constant while the amount of substrate used was reduced to one-half that used in the first experiment. The digestions with mylase P were carried out by the method of Ives and Strong (1). The results as given in Table III show that the liver enzyme plus phosphatase treatment considerably increased the apparent pantothenic acid content of the materials assayed.

DISCUSSION

One of the serious disadvantages of the use of an enzyme preparation for the liberation of a vitamin is that the blank is frequently consider-

TABLE III
*Pantothenic Acid Released from Biological
 Materials by Various Treatments*

Enzyme treatment	Liver enzyme plus phosphatase ^a		Mylase P	None
	γ pantothenic acid/g.			
	<i>A</i>	<i>B</i>		
Spinach	27.5	30.0	11.5	7.2
	21.8	23.6	11.4	7.8
Alfalfa	41.0	65.0	43.8	29.8
	46.8	58.6	37.9	26.0
Wheat germ meal	30.0	30.0	15.2	14.6
	29.3	28.6	17.2	17.3
Beef, dried	33.3	46.6	16.7	6.9
	45.6	64.6	17.3	8.9
Whole liver powder	365	400	70.0	50.4
	343	386	67.5	53.7
Yeast, dried	400	400	106	57.0
	443	486	100	58.8
Vitab	385	400	132	121
	418	436	138	130
Egg, fresh	50.7	53.3	13.0	9.9
	49.0	57.4	12.6	10.4
Sardines, Pacific ^b	15.0	12.5	6.0	5.0
	18.4	19.3	6.0	3.9
Tuna	4.2	1.1
	8.4	14.3	4.2	1.1
Sardines, Atlantic	12.0	11.5	4.7	4.1
	17.1	19.0	4.7	4.0
Mackerel, Pacific	11.3	11.0	4.7	2.7
	15.9	16.8	4.7	2.5
Salmon	15.7	5.8	4.8
	20.9	26.4	5.8	4.8
Mackerel, Atlantic	11.3	3.1	2.7
	12.0	19.3	3.1	2.4

^a In column B the substrate concentration was half that in column A. Enzyme concentrations were identical.

^b All the fish samples assayed were canned products.

ably increased. The liver enzyme solution in this work contained over 6 times as much pantothenate as the intestinal phosphatase. In the case of some of the low potency materials, the enzyme blank amounted to nearly half of the total pantothenate found after digestion. It is obvious that the blank should be reduced if this technique is to be used for the accurate, routine determination of the vitamin. Although very labile, it is possible that the liver enzyme may be fractionated or purified to remove the high level of pantothenic acid. Another approach would be to search for the liver enzyme in some material which contains less pantothenic acid.

These results emphasize that a large portion of the pantothenic acid of tissues exists in a bound form not previously included in most of the reported assay values. This may account for the higher values sometimes found in the chick assay, which fact has already been mentioned above. The suggested procedure should permit a much closer estimation of the total pantothenate content of biological materials than has been possible by previous methods.

SUMMARY

Greater liberation of pantothenic acid from a variety of biological materials was obtained by digesting the samples with a chicken liver enzyme-intestinal phosphatase mixture than by the conventional mylase P digestion. Chicken liver was found to be of equal value to pigeon as a source of the liver enzyme. The treatment is based on the method of hydrolysis of coenzyme A discovered by Lipmann and coworkers (5).

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The Distribution of Mannans in the Wood of Slash Pine and Black Spruce

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INTRODUCTION

Recently, Wise, Ratliff and Browning (1) described in detail a modification of the Hägglund-Bratt method (2) for the determination of mannans in wood. In brief, this technique depends on hydrolysis of the carbohydrates of the cell wall by use of sulfuric acid, neutralization of the hydrolyzate by barium carbonate, and evaporation of the filtrate to a point at which the mannose can be quantitatively precipitated as the insoluble phenylhydrazone. Using this same analytical procedure in the present study, the distribution of mannose units over various hemicellulose fractions and α -cellulose was determined in the case of black spruce (*Picea mariana*) and slash pine (*Pinus caribaea*). Thus, a "mannan balance" was obtained and, from this, it became evident that mannose units persisted in the α -cellulose. Furthermore, the hydrolysis and acetolysis of this α -cellulose indicated that these mannose units were still present in the more difficultly hydrolyzable fractions of the wood cellulose, and that the mannans belong largely to the most resistant portions of the cell wall, rather than to the more easily hydrolyzable hemicelluloses.

EXPERIMENTAL

Distribution of Mannans in Slash Pine and Black Spruce

The two woods, slash pine (*Pinus caribaea* Morelet) and black spruce (*Picea mariana*), were chosen because of their great economic importance. The wood meal (40–60 mesh) used in either case represented a well mixed composite sample obtained from disks cut from a number of different bolts of wood. The material was largely sapwood. Inasmuch as small amounts of mannose-yielding material might be found among the extractives, mannose determinations were made on samples of unextracted as well as on extractive-free wood. All such determinations were made by the modified Hägglund-Bratt procedure (1). The extractions were carried out with alcohol-benzene,

followed by the use of hot water. The mannan content, however, was computed on the basis of the original, unextracted wood. With unextracted slash pine, the mannan content in triplicate determinations was found to be 9.9, 9.9, and 10.1%, whereas the extracted wood showed a mannan content of 9.9 and 10.3 in duplicate runs. Within the errors of the mannan procedure, these results are in satisfactory agreement, and any mannose-yielding material removed by solvents must have been slight. Analogous determinations on black spruce showed similar results. The total mannan content of spruce was shown to be 10.3% for unextracted wood, and 10.2% for extracted wood (calculated on the basis of unextracted wood). Here too the differences are within the experimental error of the method.

The two wood samples were converted into holocellulose by the chlorite method of Wise, Murphy and D'Addieco (3) and fractionated in accordance with their procedure by means of aqueous potassium hydroxide *under nitrogen*. In each case, the hemicellulose fractions were recovered quantitatively. However, instead of 2 hemicellulose fractions, 3 fractions were isolated: hemicellulose "A" (removed by means of 5% potassium hydroxide), hemicellulose "B" (removed by subsequent treatment of the residue from A by means of 16% potassium hydroxide), and hemicellulose "C" (removed from the residue from B by subsequent treatment with 24% potassium hydroxide). The final fibrous residue was termed " α -cellulose."

The following table (I) shows the results of these determinations, based, in either case, on the original, unextracted wood.

TABLE I
Fractions Isolated from Coniferous Woods
(Calculated on the basis of unextracted wood)

Fraction	Slash pine per cent	Black spruce per cent
Hemicellulose "A"	10.5	12.8
Hemicellulose "B"	2.5	3.5
Hemicellulose "C"	1.67	0.8
α -Cellulose	50.3	49.2

In both woods, mannan determinations were made on each of the 4 fractions. The results are given in Table II.

TABLE II
Mannan Content of the Various Components of Slash Pine and Black Spruce
(Calculated on the basis of each component)

Fraction	Slash pine per cent	Mannan	Black spruce per cent
Hemicellulose "A"	11.5		9.3
Hemicellulose "B"	27.2		26.4
Hemicellulose "C"	45.2 ^a		44.9
α -Cellulose	12.2		11.9

^a Single determination; all other determinations were run in duplicate.

The mannan content of the components was then calculated on the basis of the original unextracted wood. These yields and their summation are given in Table III and are compared with the overall mannan yield found in the original wood.

TABLE III
Mannan Content of Coniferous Wood Fractions
(Based on the original unextracted wood)

Fraction	Slash pine per cent	Mannan Black spruce per cent
Hemicellulose "A"	1.3	1.2
Hemicellulose "B"	0.7	0.9
Hemicellulose "C"	0.8	0.4
α -Cellulose	6.1	5.9
Summation	8.9	8.4
Original, unextracted wood	10.0	10.3

These summations indicate that the mannan recoveries computed from the various fractions of slash pine and black spruce are about 89 and 82%, respectively. This loss in mannan may be due in part to inherent weaknesses in the mannose determination (*e.g.*, more rapid destruction of mannose freed from the hemicellulose fractions than from the more resistant wood). However, they may be due in part to losses of small amounts of mannan during the isolation of holocellulose and in the recovery of the hemicellulose fractions.

There is, however, another possibility. Earlier experiments at the Institute (1) showed that, whereas glucose appears to aid in the mannose determination, xylose impedes the recovery of mannose. This inhibitory effect is noticeable especially when the mannose concentrations are low.

In two recent experiments, a similar effect was noted under somewhat different conditions. Approximately 0.45 g. of mannose, 0.45 g. of xylose, and 1.8 g. of glucose were added to 72% sulfuric acid, diluted immediately with water to form a 3% acid solution, heated just to the boiling point, and neutralized directly with barium carbonate. Otherwise, the mannose determination was carried by the usual procedure (1), even to the addition of a small (0.15 g.) weighed booster charge of mannose just prior to the precipitation. The mannose recoveries were 83.0 and 83.1% of the amounts taken. Therefore, it may well be that the xylan present in hemicellulose fractions is instrumental in lowering mannose yields. In the original slash pine, the total pentosan content calculated as xylan was only 8.25%. This would have a minor effect on the mannose determination, because of the predominantly large amount of glucose present (due to hydrolysis of the cellulose of the wood).

On the other hand, the xylan content in hemicellulose "A" of slash pine was found to be 54.3%. This might well have a disturbing effect on the mannose determination, despite the addition of a sufficient booster charge to bring the mannose content to about 1% prior to its precipitation, and would account for a lowered mannan content in the hemicelluloses and the lower summation given in Table III.

In the mannan analyses cited above, the mannan content of the coniferous woods is appreciably higher than those reported for these same samples in a previous publication (4). This increased mannan content appears to be due to refinements in the determination made since the older analytical data were obtained. It is quite possible, and even probable, that the mannan data given for other species (3,4) also represent minimal values.

TABLE IV
*Cu₂O Reducing Values Found in Successive Hydrolyzates from
Two Samples of Slash Pine [Alpha + C]*

Number of 1-hr. intervals	Cu ₂ O from hydrolyzates representing 0.500 g. material, mg.	
	Sample I	Sample II
1	69.9	70.3
2	25.1	26.0
3	16.4	15.3
4	14.3	14.3
5	10.9	10.5
6	10.7	10.1
7	7.6	6.1
8	6.8	8.7
9	5.1	8.2
10	5.0	3.0
Summation	171.8	172.7

The mannan determination (1) as outlined above still leaves much to be desired. The hydrolytic procedures remain unsatisfactory. The analyst finds himself between the Scylla of a "brutal" treatment of plant tissues with strong sulfuric acid and the Charybdis of an incomplete hydrolysis of mannan when more dilute acids are used.

*Hydrolysis of the α -Cellulose Fraction of
Slash Pine ["Alpha + C"]*

Inasmuch as hemicellulose "C" referred to above was a small, highly insoluble fraction (1.67% of the wood), the following experiments were carried out with the

fibrous residue retaining this material. This composite sample contained 13.4% mannan (in contrast to 12.2% in the α -cellulose freed from "C"). For the sake of brevity, this fibrous material was termed ["Alpha + C"].

Two 1 g. samples of [Alpha + C] were treated for 1 hr. at 98–9°C. with 50 ml. of 5% sulfuric acid, using an Erlenmeyer flask fitted with an air condenser. The hydrolyzate was removed by filtration, the residue washed with small amounts of water, and

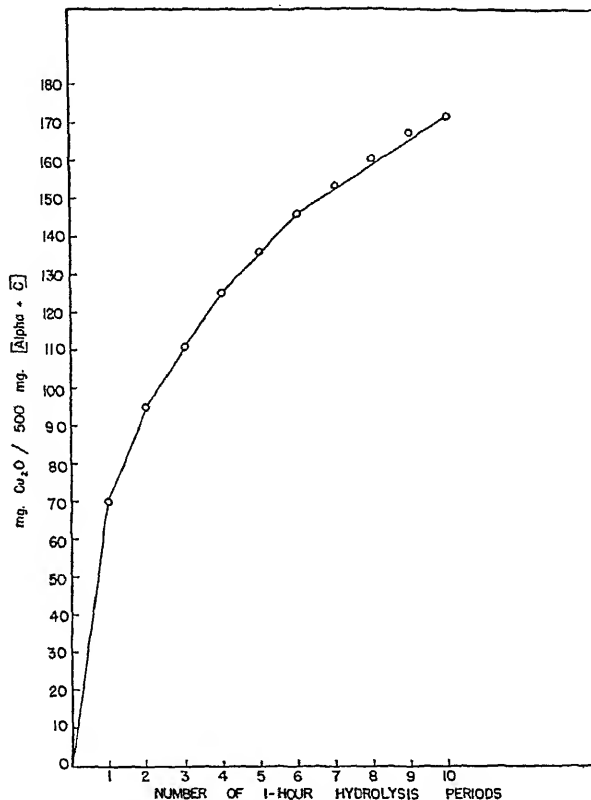


FIG. 1. Hydrolysis-time curve of slash pine alpha-cellulose (containing hemicellulose "C").

then returned to the reaction flask for further hydrolysis with a fresh 50-ml. portion of 5% acid. Ten such successive 1-hr. hydrolyses were made. In each case, the combined filtrate and washings from the cellulose residue were diluted to 100 ml., filtered, and neutralized with solid sodium carbonate. Fifty-ml. aliquots of each neutral filtrate were analyzed for reducing sugars by the standard Munson-Walker procedure. Results are given in Table IV.

The final residue was a gel, difficult to filter, which, on careful drying (after washing with alcohol and ether), was obtained as a finely divided powder typical of a hydro-cellulose, and showing none of the physical appearance of the original [Alpha + C]. A hydrolysis time curve is shown in Fig. 1. Here the reducing value (mg. $\text{Cu}_2\text{O}/500$ mg. material) has been plotted against number of successive hydrolyses. This shows graphically the changes indicated in Table IV.

That mannose units were retained tenaciously in the powdery residue, even after extensive hydrolysis, is shown in Table V. The experiments were carried out by heat-

TABLE V
*Mannan in Slash Pine Cellulosic Residues after
Various Stages of Hydrolysis*

Sample [Alpha + C]	Mannan based on original material per cent
Unhydrolyzed	13.4 ^a
Residue after 1 hr. hydrolysis	9.9 ^a
Residue after 2 successive 1 hr. hydrolyses	10.0 ^b
Residue after 8 successive 1 hr. hydrolyses	7.5 ^a

^a Average of duplicate determinations.

^b Single determination.

ing the [Alpha + C] sample with 5% sulfuric acid (50 ml./g.) for successive 1-hr. periods and, after certain intervals, washing and drying the residues, which were then hydrolyzed with 72% sulfuric acid and analyzed for mannose by the usual procedure. Even after 8 successive hydrolyses with fresh charges of 5% sulfuric acid, the hydro-cellulose residue still retained over 55% of the mannan content of the original fibrous material [Alpha + C] and almost 40% of that present in the original wood. It is evident that other sugars besides mannose are lost during the hydrolysis.

These results are in harmony with those obtained by previous investigators (5,6) who have emphasized the resistance of a part of the mannans in wood to alkaline extraction and to acid attack.

Acetolysis of Slash Pine α -Cellulose

In an effort to determine whether sugars containing mannose units could be obtained from slash pine α -cellulose, 5 g. of this material (*freed from hemicellulose "C"*) were acetolyzed by Spencer's method (7). The acetolysis was carried out at 50°C. over a 14-day period, using the optimal quantities of acetic anhydride and sulfuric acid recommended by Spencer, and working up the products in accordance with his techniques. However, all mother liquors from the principal crystalline products were evaporated, and the products hydrolyzed and tested for mannose. It was hoped that a saccharide containing one or more mannose units might be isolated, but none of the crystalline portions yielded mannose; only the so-called celloextrin acetates gave mannose on hydrolysis. Details of this experiment need not be given.

The results, however, can be summarized as follows: the yields of the 3 main fractions were (I) crude cellobiose octaacetate, 3.13 g.; (II) crude glucose pentaacetate, 1.177 g.; (III) cellodextrin acetates, 3.52 g. On hydrolysis, the latter yielded appreciable amounts of mannose phenylhydrazone. (I) on recrystallization and fractionation gave only pure octaacetyl- α -cellobiose, $[\alpha]_D^{25}$ 41.7° (CHCl_3), m. 227.5°C., and (II) yielded only pentaacetyl- α -D-glucose, $[\alpha]_D^{25}$ 97.7° (CHCl_3), m. 112–3°C. Mixed melting points with authentic samples in either case showed no melting point depressions.

The fact that mannose units reside in the longer chain cellodextrins confirms the hypothesis that a portion of the mannose should be considered a part of coniferous wood cellulose, rather than belonging to the less stable hemicelluloses. The acetolysis studies will be continued with larger samples.

DISCUSSION

As a result of this study, it is evident that, in coniferous woods, the hemicellulose fraction most readily removed by alkali (*i.e.*, that highest in polyuronides) contains the lowest amount of mannan, whereas the mannan content increases in other hemicellulose fractions with decreasing alkali solubility. This is in harmony with previous unpublished findings of Anderson, and the analytical group of The Institute of Paper Chemistry (8) on Douglas fir. If a true (homopolymer) mannan actually occurs in wood, these less soluble hemicellulose fractions should form a starting material for its isolation.

As evidenced both by hydrolysis and acetolysis experiments, an important portion of the mannan in wood must be considered an integral portion of the most resistant cellulose. Thus, Bertrand's assumption of a "mannocellulose" (9) finds experimental justification.

ACKNOWLEDGMENT

Pyridose (pyridylmercuric acetate), supplied by Mallinckrodt Chem. Works, and highly purified sodium chloride, donated by Mathiesen Chem. Corp., are gratefully acknowledged.

SUMMARY

The distribution of mannans over the various hemicellulose fractions of slash pine and black spruce, respectively, was studied. In either wood, the fraction most readily extracted by alkali contained the lowest

percentage of mannan, whereas the least soluble fraction showed the highest mannan content. Over half the mannose units of these two woods, however, was retained in the resistant α -cellulose residue. A study of the "mannan balance" obtained through the summation of the individual mannan contents of each fraction of these two softwoods (calculated on the original wood basis) accounted for 82-89% of the mannan present in the original wood. Even after repeated hydrolysis of slash pine α -cellulose with 5% sulfuric acid, the hydrocellulose residues still retained appreciable amounts of mannan. Acetolysis of slash pine α -cellulose indicated that mannose units persist in the acetylated cello-dextrin fraction. It is evident that a large part of the mannan originally present in the wood cannot be classed with the alkali-soluble, easily hydrolyzable hemicelluloses, and that the term "mannocellulose" is justified experimentally.

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16-C¹³-Dehydroisoandrosterone Acetate

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INTRODUCTION

During the last decade physiologists have been increasingly interested in the genesis and fate of the steroid hormones in the body. Such investigations could be undertaken more efficiently if there were available hormones of the steroid series containing tagged carbon atoms (either C¹³ or C¹⁴)¹ in the cyclopentanophenanthrene ring system. Turner recently announced the synthesis of cholestenone (1) and testosterone (2) containing radiocarbon C¹⁴ at position 3, and MacPhillamy² has prepared methyltestosterone with C¹⁴ in the methyl group on carbon atom 17.

It appeared to us that it would be advantageous to work out a general method which would be useful in both the androgen and the estrogen series. For this purpose it was necessary to introduce the tagged carbon atom in one of the rings other than the A ring of the steroid nucleus.

The method outlined³ by Kuwada and Nakamura (3) for the synthesis of dehydroisoandrosterone acetate (IX), from which testosterone is readily accessible, suggested a possible means to this end. These authors reformed ring D of the steroid nucleus starting with 3-hydroxyetiobilienic acid (I). For the estrogen synthesis one would have to use the analog of (I) with an aromatic A ring which can be obtained by opening the D ring of estrone methyl ether (4), or by total synthesis (compare 5,6).

¹ At the time we started our experiments, radioactive C¹⁴ was difficult to obtain and we used instead the more readily available C¹³ which was supplied as potassium or sodium cyanide. We are indebted to Drs. C. P. Rhoads and K. Dobriner of the Sloan-Kettering Institute for Cancer Research, New York, for the supply of cyanide containing heavy carbon. Undoubtedly, the procedures described in this paper will work as well with KC¹⁴N which may be prepared readily from BaC¹⁴O₃ (15).

² Private communication.

We prepared dehydroisoandrosterone acetate containing the heavy isotope C^{13} by the scheme outlined in Formulas (I) to (IX). The acetic acid side chain in etioobilienic acid was lengthened by one heavy carbon atom using the Arndt-Eistert procedure and then the D ring of the sterol ring system was formed by pyrolysis of the anhydride of the acid thus obtained by using Blanc's method. The question arose, therefore, whether in this completely aliphatic system the tracer carbon atom added to the acetic acid side chain would appear as the terminal carboxyl group or as the adjoining methylene group of the homo acid. If it became the carboxyl group it would probably be expelled as carbon dioxide in the thermal cyclization process. As the methylene group, it would appear as carbon atom 16 in the product after cyclization.

The product of the reaction contained the calculated amount of the heavy isotope.³ There is, therefore, no doubt that also in this case the Arndt-Eistert reaction involves a rearrangement so that the tracer carbon atom appears as the methylene group of the homo acid. This course of reaction is, therefore, in agreement with the mechanism as elucidated by Huggett, Arnold and Taylor (7) using isotope C^{13} , who showed that, in the case of benzoic acid, the carbon atom introduced by the Arndt-Eistert procedure appeared in the final product in the methylene group adjoining the carboxyl group of the phenylacetic acid produced. Later, Dauben, Reid, Yankewich and Calvin (8) repeated this work using radiocarbon C^{14} and obtained identical results.

The requisite intermediate 3-hydroxy- Δ^5 ,⁶-etioobilienic acid, (I), was obtained both from the acid fraction resulting from the oxidation of dibromcholesterol acetate with chromic acid⁴ and from the alkaline hypiodite oxidation of dehydroisoandrosterone acetate (10,11,12). In the case of the latter reaction, we found that it was unnecessary to keep the iodine in excess during the oxidation as recommended by previous workers (10). Furthermore, we observed that a too rapid addition of the iodine solution caused the precipitation of an unstable and very slightly soluble iodo derivative of dehydroisoandrosterone acetate. Its formation apparently was catalyzed by the presence of a large area of stainless steel in the form of a cooling coil; and the best results in the

³ The mass spectrograph determinations of heavy carbon were kindly performed for us by Professor D. Rittenberg and Dr. David B. Sprinson of the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York.

⁴ Dr. Bradley Whitman and Mr. Temple Clayton of this laboratory prepared a large part of this starting material by an improved isolation procedure.

oxidation were obtained when the reaction was conducted in an all-glass apparatus. Once formed, the iodine compound reacted very slowly with alkali and the yield of acid was proportionately lower. Upon isolation, this by-product proved to be unstable and lost iodine upon standing. Freshly prepared samples showed an iodine content of about 38%, whereas the diiodo compound should contain 43.7% and the monoiodo compound 27.9%. Solutions in methanol were colored deep brown by liberated iodine and, after 5 recrystallizations, analysis showed an iodine content of 32.4%. Upon reduction with zinc dust in hot acetic acid, dehydroisoandrosterone acetate was regenerated. This behavior is consistent with Formula (X).

The bulk of the product of the oxidative ring cleavage appeared to be the C-17, or "β"-methyl ester of etiobilienic acid, but occasionally the dimethyl ester, (II), was also formed in small amount, thus confirming the findings of earlier workers (10).

The formation of esters is unexpected in view of the fact that the reaction is carried out in alkaline solution. To explain the observed facts, one may assume that, in analogy with the known course of the haloform reaction (13,14), the first step in the degradation of dehydroisoandrosterone acetate is the substitution of iodine in the C-16 position. Both mono- and diiodo derivatives may be formed. When the addition of iodine is rapid, or is catalyzed, the rate of formation of the diiodo compound exceeds that of its conversion into etiobilienic acid esters, and hence it precipitates without ring cleavage. If, however, a slower pace of addition of iodine is maintained, the iodo compound does not reach saturation and cleaves with the addition of the elements of methanol between carbon atoms 16 and 17 to produce the dimethyl ester. The latter, however, is quite easily hydrolyzed in the cold, and for this reason the 17-monomethyl ester, (III), is the main product of this reaction. It was possible to isolate the 17-monomethyl ester in good yield as described in the experimental part.

Diazomethane containing the isotope C¹³ for use in the Arndt-Eistert reaction has been prepared for the first time from potassium or sodium cyanide. Samples of cyanide containing from 3 to 24% of C¹³ were reduced to methylamine with chromous chloride (8), the latter being conveniently prepared by electrolytic reduction, in dilute acid solution, of the readily available green chromic chloride (9).

Cyclization [Blanc's reaction, (VIII-IX)] by pyrolysis of the acetoxy 16-homo-acid anhydride prepared in acetic anhydride solution gave

erratic and poor yields of dehydroisoandrosterone acetate. However, an increase from an average of 25% to consistent yields of 47% was obtained by treating the acid with a relatively small amount of acetic anhydride in acetic acid solution in order to prepare the anhydride. In the former case, a large residue of acidic resin was left in the pyrolysis flask and it is possible that the formation of mixed anhydrides with acetic acid was responsible for the poor results.

EXPERIMENTAL^{5, 6}

3(β)-Hydroxyetiobilenic Acid from Dehydroisoandrosterone Acetate

The methanol used in this experiment was pretreated with alkaline hypoiodite at room temperature in the proportion of 200 g. of iodine and 250 g. of potassium hydroxide to 15 l. of methanol. After a negative test was obtained with moist starch-iodide paper, the methanol was distilled from a water bath. In the oxidation of dehydroisoandrosterone acetate, the addition of the iodine solution was divided into two stages, the first and larger portion paralleling the addition of alcoholic alkali, while the second portion was added simultaneously with the aqueous alkali. Under the conditions of the experiment, a considerable excess of alkali over that required by the iodine was always present.

A solution of 50.0 g. of dehydroisoandrosterone acetate (0.151 mole) dissolved in 4 l. of the pretreated methanol was placed in a flask equipped with an efficient glass stirrer and cooled to 8–12°C. by an ice-bath. To this was added at once 50 cc. of a solution of 60 g. of potassium hydroxide (85%, pellets = 0.91 mole) in 500 cc. of methanol, and the remainder was proportioned over a 2 hr. period. There was added simultaneously over the same 2 hr. period 600 cc. of a solution of 115 g. of iodine (0.455 mole) in 1 l. of methanol. The alcoholic potassium hydroxide solution was followed by a solution of 160 g. of potassium hydroxide (85%) in 1,250 cc. of water added over a second 2 hr. period. This addition paralleled that of the remaining 400 cc. of methanolic iodine solution. After the 4 hr. required to add the reagents, the solution was a clear light orange-brown color. It was allowed to stand overnight and warm to room temperature. Then the methanol was removed under vacuum at the water pump until the appearance of solid. From 1 to 2 l. of ice and water was added and the solution acidified with HCl. The precipitate was collected by suction, washed well with water, and sucked dry with a rubber dam. A dried sample melted at 201–210°C.

Purification was accomplished through the formation of the sparingly soluble potassium salt of 3-hydroxyetiobilenic acid in methanol. For this purpose, the still moist precipitate was dissolved in 700 cc. of methanol, to which 70 g. of KOH was then added. The mixture was refluxed for 2 hr. in a water bath at 75–80°C. to hydrolyze any ester which might be present. Higher temperatures caused local overheating and blackening. The thick sludge required 6–8 hr. to filter on a hardened filter paper, but

⁵ Micro analyses by Mr. Edwin Connor of this laboratory.

⁶ All melting points are corrected.

the process required little attention. The solid collected on the paper was washed several times with methanol. It was then dissolved in 1.5 l. of water and the resulting orange solution was treated with charcoal and acidified at the boiling point with HCl. The precipitated acid was collected with suction, washed with water and, while still damp, recrystallized from dilute methanol. Thick prisms were formed by seeding the hot solution and allowing the crystals to grow while evaporating the methanol and concentrating the solution. After two such crystallizations, there was obtained 31.1 g. (59%) of 3-hydroxyetiobilienic acid, m.p. 250–252°C. dec., inserted at 245°C. Further material could be obtained from the alkaline methanol mother liquors from the precipitation of the potassium salt by evaporation of the methanol, followed by acidification and conversion of the acid into the dimethyl ester with diazomethane.

Isolation of the By-Product Iodine Compound, (X)

In the early experiments, a light-yellow, insoluble precipitate occasionally formed after about half of the iodine had been added, particularly with fast addition. When a stainless steel coil which had been previously etched by use in other acidic reactions was used for cooling, the formation of this iodine compound was particularly rapid and the bulk of the precipitate separated in the space of 5–10 min. after the reaction had proceeded for about 1 hr. It amounted to nearly half of the starting material. Upon isolation, the compound was unstable; and, while it crystallized well from methanol in the form of pale yellow needles, it lost iodine slowly, both in solution and in the crystalline state.

The crude material had an iodine content of 37.7% and melted at 172–173°C. dec., while, after 5 recrystallizations from methanol, it melted at 164–166°C. dec., and the iodine content had dropped to 32.4%.

Anal. Calc'd for C₂₁H₂₈O₃I₂: I, 43.7; Calc'd for C₂₁H₂₈O₃I: I, 27.9.

A 1.00 g. sample of the crude iodine compound was suspended in 25 cc. of acetic acid and treated with zinc dust. The mixture was shaken for 10–15 min. at room temperature and then was warmed on the steam bath for 10 min. longer. After filtering the zinc dust, concentrating the solution, and diluting with water to saturation at the boiling point, there was obtained 0.43 g. (69%) of dehydroisoandrosterone acetate, m.p. 168–170°. Mixed m.p. with authentic material was unchanged.

17-Monomethyl 3-Acetoxyetiobilienate, (IV) (12)

(a) *Esterification of Diacid I with Diazomethane.* A 1.6 l. volume of methylene chloride containing 1.3 moles of diazomethane was added gradually to a suspension of 150 g. of 3-hydroxyetiobilienic acid in 500 cc. of methylene chloride, retaining 200 cc. which was added after about 1 hr., when nitrogen evolution had ceased. One hr. later the solution was filtered from unreacted acid (27.5 g.), treated with a few cc. of acetic acid to destroy excess diazomethane, and then washed with dilute sodium carbonate solution and with water. After drying, the solvent was removed by distillation. The residue of dimethyl ester (II), weighed 123 g. (93%).

(b) *Partial Hydrolysis of (II) to the 17-Monomethyl ester.* To a solution of 118 g. of dimethyl-3-hydroxyetiobilienate in 1.2 l. of methanol was added a solution of 35.5 g. of potassium carbonate in 165 cc. of water and the solution was refluxed for 1 hr. One

liter of water was added and about 1 l. of methanol was distilled, at which point the solution became turbid. Refluxing was continued until the solution became clear, whereupon water was again added until turbidity resulted. This process was repeated until 2.5 l. of water had been added over a 24 hr. period, when the remaining methanol was removed by distillation and the aqueous solution was acidified by pouring into dilute HCl. There was obtained 104 g. (99%) of monomethyl ester, m.p. 180–190°C.

(c) *Acetylation of (III)*. A solution of 106 g. of crude 17-monomethyl ester in 750 cc. of acetic acid and 250 cc. of acetic anhydride was warmed to 96–98°C. for 2 hr. and 500 cc. of water was then added gradually to decompose excess anhydride. The solution was then refluxed for a short time, after which more water was added until saturation. Upon cooling, filtering, and recrystallization of the product from dilute acetone, there was obtained a first crop of 68.1 g. (57%), m.p. 170–171.5°C.

The same compound could also be obtained directly from the hypoidite oxidation of dehydroisoandrosterone acetate. In one experiment, 39 g. of crude oxidation product was acetylated with a mixture of 300 cc. of acetic acid and 100 cc. of acetic anhydride by warming for 2 hr. on the steam bath. After decomposing the excess acetic anhydride and adding water to saturation at the boiling point, there was obtained 34.5 g. of product, m.p. 155–160°, the melt still showing unchanged solid at 200°. Recrystallization from dilute acetone gave 32 g. of 3-acetoxyetiobilienic acid 17-methyl ester, m.p. 167–172°C.

3-Acetoxyetiobilienic-16-Acid Chloride 17-Methyl Ester, (V)

A solution of 50 g. of the 17-methyl ester of 3-acetoxyetiobilienic acid in 100 cc. of purified thionyl chloride was allowed to stand at room temperature for $\frac{1}{4}$ hr. and the thionyl chloride then removed at the water pump while warming the flask in a bath at 45°. Dry benzene was added to the crystalline residue and the vacuum distillation was repeated to remove traces of thionyl chloride. The acid chloride was then dissolved in 1.5 l. of dry hexane at the boiling point and filtered from 4.0 g. of unchanged acid. After concentrating the solution to 500 cc. and cooling, there was obtained 48 g. (100%) of acid chloride m.p. 136–137°C. Heer and Miescher (12) report a m.p. of 135°C. in a small scale experiment using oxalyl chloride instead of thionyl chloride.

3-Hydroxyetiobilienic Acid 16-Methyl Ester

To a solution of 6.8 g. of 3-hydroxyetiobilienic acid dissolved in 75 cc. of methanol was added 0.75 cc. of acetyl chloride. After 24 hrs. at room temperature, the methanol was evaporated under vacuum, and the dry crystalline residue upon recrystallization from acetone-benzene gave 6.2 g. (88%), m.p. 181–183°C. Another crystallization raised this melting point to 183–184°C., $[\alpha]_D^{25} = -57.3$ (absolute alcohol). Kuwada and Nakamura (3) report the m.p. to be 186.5–188°C., $[\alpha]_D^{25} = -55.9^\circ$.

Sodium-Alcohol Reduction of the 16-Ester to the 16-Carbinol⁷

A solution of 1.75 g. of 16-monomethyl-3-hydroxyetiobilienate was dissolved in 150 cc. of *n*-butanol and the solution was distilled to a volume of 125 cc. This nearly

⁷ We are grateful to Drs. Harold Levy and Robert P. Jacobsen of the Worcester Foundation for Experimental Biology, in Shrewsbury, Massachusetts, for calling our

boiling solution was quickly added to 20 g. of sodium which had been freshly cut and washed several times with dry butanol. After refluxing for $\frac{1}{2}$ hr. over a bath at 125°C. water was added in portions until the excess sodium had dissolved. Steam distillation of the butanol was followed by pouring the solution into an excess of dilute HCl. After collection of the solid, washing with water, and drying under vacuum there remained 1.78 g., m.p. 124–129°C. Recrystallization from dilute acetone gave a product which, upon slow heating, softened at 120°C., then resolidified and melted at 185–195°C. Four recrystallizations from ethyl acetate raised the melting point to 207–209.5°C., the carbinol evidently losing water easily to form the cyclic lactone. Analysis indicated a half molecule of ethyl acetate of crystallization.

Anal. Calc'd for $C_{19}H_{28}O_3 \cdot \frac{1}{2}C_4H_8O_2$; C, 72.37; H, 9.27.

Found: C, 72.17; H, 9.43.

This material was acetylated to 3-acetoxyetiobilienic-17-acid-16-carbinol lactone by refluxing 0.46 g. of the lactone with a few cc. of acetic anhydride for 1 hr. Upon removing the solvent under vacuum there remained a residue which, after recrystallization from acetone-ligroin, formed 0.40 g. of flat needles, m.p. 186–188°; $[\alpha]_D^{25} = -65.1^\circ$ (dioxane) unchanged after 5 recrystallizations from acetone.

Anal. Calc'd for $C_{21}H_{30}O_4$; C, 72.78; H, 8.73.

Found: C, 73.00, 72.91; H, 8.60, 8.72.

attention to the fact that this substance should be isomeric with their dehydroisoandrolactone (16). They were kind enough to make melting point determinations of mixtures of both hydroxy lactones and of both lactone acetates. The following results were obtained:

	<i>M.P.</i>	<i>Rotation</i> (dioxane)
Our hydroxy lactone (see above)	207.5–209°	
Dehydroisoandrolactone of Levy and Jacobsen	239.5–243.5°	$[\alpha]_D^{25} - 254.7^\circ$
Mixture	Softens 185°, melts 210–226.5°	
Our lactone acetate (see above)	185–187°	$[\alpha]_D^{25} - 65.1^\circ$
Dehydroisoandrolactone acetate of Levy and Jacobsen	183.5–185°	$[\alpha]_D^{25} - 109.4^\circ$
Mixture	Softened upon insertion at 147°, melts 149.5–151.5°	

The rotations listed above on dehydroisoandrolactone and its acetate were determined on samples sent to us by Drs. Levy and Jacobsen. There is no doubt that the two compounds are different.

*Methylamine Hydrochloride by Chromous Chloride
Reduction of Potassium Cyanide*

Chromous Chloride was prepared by the electrolytic reduction of green chromic chloride hydrate in dilute hydrochloric acid solution according to the procedure of Traube, Burmeister and Stahn (9).

A solution of 83 g. of $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$ (0.31 moles), 3 cc. of conc. hydrochloric acid and 120 cc. of water was placed in a porous cup with a 70 cm.² electrolytic lead cathode, covered with a layer of ligroin, and electrolyzed for 18–19 hours with a current of 0.6–0.7 amp. At the end of this time, titration with iodine showed that 0.24 moles (80%) of chromous chloride was present. Slight variations in the condition of the electrode surface and the current density varied the yield from this value to nearly quantitative.

Methylamine Hydrochloride (9)

A five-necked flask was arranged to carry a glass electrode and a potassium chloride bridge with calomel half-cell connected to a Coleman pH electrometer through two openings near the surface of the liquid, using rubber tubing to connect to the bakelite fittings. The other openings held a pressure equalized dropping funnel, a platinum wire stirrer and a connection to a condenser arranged for down distillation into a two-necked flask as a receiver. Hydrogen gas was used as an inert atmosphere and the methylamine solution produced was condensed in the second flask, to which was attached a spiral tube wash bottle containing dilute HCl, which acted as a safeguard and through which the hydrogen was vented. Sufficient acid was used to later neutralize the methylamine formed in the reaction. Into the flask was placed 2.3 g. of 93.2% sodium cyanide containing 2.0 at.-% excess C^{13} and to it was added a cold solution of 15 g. of sodium hydroxide in 100 cc. of water and the flask was then cooled in an ice bath. The chromous chloride solution (30% excess) prepared above was added rapidly with stirring and in an atmosphere of hydrogen. The pH at this point was 7–7.5 and the solution was warmed to 80–85°C., the mixture forming a green precipitate. Reduction was complete after about 1 hr., whereupon the electrodes were removed. The openings were stoppered, 20 g. of sodium hydroxide in 100 cc. of water was added and the methylamine was distilled by heating to boiling with continued stirring. After about 100 cc. of aqueous distillate had been collected, the dilute acid in the wash bottle was emptied into the methylamine solution in the receiver. The solution was transferred to a distillation apparatus, and, while warming on a steam bath, the water was removed under water pump suction until a dry residue remained in the flask. This residue was extracted with hot *n*-butanol, concentrated to a volume of about 100 cc., filtered to remove undissolved ammonium chloride and chilled to crystallize the amine salt. The vacuum-dried crystalline methylamine hydrochloride weighed 1.09 g. (37%). In preparative experiments the methylamine hydrochloride was not purified with resulting loss due to crystallization, but was used in admixture with the ammonium chloride to prepare nitrosomethylurea and diazomethane.

Anal. At.-% excess C^{13} ; Calc'd, 2.0. Found, 2.06.

Nitrosomethylurea

Crude methylamine hydrochloride from 3 successive reductions totaling 6.68 g. of 93.2% sodium cyanide was refluxed for 3 hr. with 25.5 g. of urea in 33 cc. of water. The

solution was chilled and 9.3 g. of sodium nitrite was then dissolved in it. A mixture of 51 g. of ice and 9.3 g. of sulfuric acid was then added slowly and with stirring, the temperature being held at -5 to -10°C . The nitrosomethylurea was collected and dried under vacuum in a desiccator over barium oxide. Weight, 11.9 g.

Diazomethane

Crude nitrosomethylurea from the above preparation was hydrolyzed to diazomethane by adding it in small portions to a stirred mixture of 125 cc. of ether and 18 cc. of 50% KOH solution cooled in an ice bath. After stirring for $\frac{3}{4}$ hr. the diazomethane solution in ether was decanted. The residue was then triturated with 15 smaller portions of ether and the ether extracts combined and dried over pellets of KOH. The diazomethane content of the ether solution, determined by adding an excess of benzoic acid to a 1 cc. sample and then back-titrating with alkali, was found to be 0.0378 moles (30% based on the sodium cyanide used).

3-Acetoxyetiobilienic-16-Homoacid Amide 17-Methyl Ester, (VII), from the 16-Acid Chloride by the Arndt-Eistert Reaction

To a solution of 0.015 mole diazomethane (prepared by reduction of KCN containing 22.70 at.-% excess C¹³) dissolved in 65 cc. of anhydrous ether, was added 2.07 g. (0.005 moles) of the 16-acid chloride dissolved in 70 cc. of ether. The solution was allowed to stand overnight at -5°C . and the solvent then removed at the water pump. The residue was dissolved in 170 cc. of 95% ethyl alcohol warmed to 50 – 55°C . and a solution of 0.9 g. of silver nitrate in 17 cc. of concentrated aqueous ammonia was added. The dark mixture was refluxed for 1.5 hr. It was then concentrated to small volume, diluted with water at the boiling point until a slight turbidity persisted, and 1 cc. of saturated sodium chloride solution together with 1–2 g. of decolorizing charcoal was added. After filtering by gravity, water was added until the solution was saturated and it was allowed to cool slowly, whereupon the homoamide crystallized in the form of leaflets. There was obtained 1.7 g. of material, m.p. 161 – 163.5°C . (83% theory). M.p. reported (3), 165 – 166°C . In larger scale experiments yields varying from 60–77% were obtained.

Anal. At.-% excess C¹³: Calc'd, 0.987; Found, 0.980.

16-C¹³-Dehydroisoandrosterone Acetate, (IX), from 3(β)-Acetoxyhomoetiobilienic Anhydride

Hydrolysis of the amide (obtained by the above procedure but using sodium cyanide containing 2.0 at.-% excess C¹³) was effected by refluxing 0.62 g. for 1 hr. with 10 cc. of methanol and 1 g. of potassium hydroxide. Then 10 cc. of water was added, the aqueous solution was refluxed overnight and poured into cold dilute acid. Recrystallization of the 16-homo acid from dilute methanol gave 0.44 g. (80%) of material, m.p. 248 – 249.5°C .

Following the directions of Kuwada and Nakamura (3), a 1.87 g. sample of acid thus prepared was converted into the 3(β)-acetoxyhomoetiobilienic anhydride and subsequently pyrolyzed to dehydroisoandrosterone acetate by heating at 250 – 265°C . for 15 min. in a flask with a sealed-on receiver at about 15 mm. pressure. Finally, the product was distilled at a pressure of 0.5 – $2\ \mu$. The yield of ketone isolated as the semi-

carbazone from methanol solution varied from 18 to 32%. There remained in the pyrolysis flask an acidic resin which dissolved in dilute alkali but which did not give dehydroisoandrosterone upon repetition of the process of anhydride formation and pyrolysis.

A sample of 200 mg. of 16-C¹³-dehydroisoandrosterone acetate, m.p. 168–169.5°C. (pure dehydroisoandrosterone acetate melts at 170.2–170.8°C.), obtained by hydrolysis of 307 mg. of the semicarbazone in dioxane with sulfuric acid gave the following analysis:

Anal. At.-% excess C¹³: Calc'd, 0.095. Found, 0.104.

In later experiments, using homoacid prepared from ordinary potassium cyanide, it was found that the formation of the anhydride was improved by using a dilute acetic acid-acetic anhydride solution. For example, 0.500 g. of 3-hydroxy-16-homoetiobilienic acid was refluxed for 10 hr. in a solution of 30 cc. of acetic acid and 2 cc. of acetic anhydride and the main body of the solvent removed by distillation at atmospheric pressure, applying suction to remove the last traces. The residue when processed as above consistently gave 0.27 g. (47%) of dehydroisoandrosterone acetate semicarbazone. There still remained some high boiling resin in the pyrolysis flask.

SUMMARY

The preparation of 3-hydroxyetiobilienic acid by hypiodite oxidation of dehydroisoandrosterone has been studied and a new intermediate iodine compound has been isolated.

The preparation of diazomethane containing the isotope C¹³ has been described.

16-C¹³-dehydroisoandrosterone acetate has been synthesized.

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Conversion of Pteroylglutamic Acid to Pterioic Acid by Bacterial Degradation

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INTRODUCTION

In the course of an experiment in which a sample of pteroylglutamic acid (PGA) was stored in a pH 7.0 buffer solution, a precipitate developed which was identified as pterioic acid (1). It was suspected that this degradation of pteroylglutamic acid to pterioic acid was caused by bacterial action. This paper describes the isolation and classification of the organism involved and some observations on its nutritional requirements. The isolation and crystallization of pterioic acid is outlined.

EXPERIMENTAL AND RESULTS

Inhibitors of the Degradation

To ascertain whether the degradation reaction was of a biochemical nature, a 0.4% solution of PGA in *M*/5 phosphate buffer was incubated, after inoculation from a flask exhibiting the characteristic turbidity, under the conditions outlined in Table I.

The presence of phenol, chloroform or toluene, or sterilization by heating on the steam bath, completely inhibited the formation of pterioic acid. These results suggested that the conversion of pteroylglutamic acid into pterioic acid under the experimental conditions was due to the action of a microorganism.

Isolation of the Organism Splitting PGA

When the organisms from an actively fermenting solution were plated in a medium consisting of *M*/5 pH 7.0 phosphate buffer, 0.5% PGA and 1.5% agar, yellow and bluish-white colonies developed in 36–48 hours. Cultures were picked from representative type colonies and transferred to agar slants of the above composition.

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Inoculation of *M/5* pH 7.0 phosphate buffer containing 0.5% PGA with each organism, and as a mixed culture, showed the yellow pigmented organism to be responsible for the splitting of pteroylglutamic acid.

TABLE I
Inhibitors of the Degradation

Conditions	Appearance after			
	52 hr.	69 hr.	74 hr.	118 hr.
1. Control	Ppt.	Ppt.	Ppt.	Ppt.
2. 0.1% Phenol added	Clear	Clear	Clear	Clear
3. Chloroform added	Clear	Clear	Clear	Clear
4. Toluene added	Clear	Clear	Clear	Clear
5. pH 7 Buffer heated in the steam bath 15 min. before addition of PGA	Clear	Clear	Ppt.	Ppt.
6. PGA solution heated 15 min. on steam bath	Clear	Clear	Clear	Clear
7. 0.15% Glutamic acid added	Sl. turbid	Ppt.	Ppt.	Ppt.

Identification of the Organism

Morphology. A 24-hr. culture grown on the phosphate buffer-PGA-agar medium at 30°C. when stained with a solution of nigrosin showed very short rods 0.5–1.0 μ in length. A 48-hr. culture and a one-week culture showed the same size rod.

Staining Reactions. The organism is Gram-negative. Welch's capsule staining of 24- and 48-hr. cultures did not show the presence of capsules. Staining with methylene blue failed to demonstrate the presence of polar granules or barred forms. Nigrosin stain is the only stain that distinctly shows the rod-shaped nature of the bacterium.

Spore Formation. Heat shock treatment was done using a suspension of the organism in saline at 80°C. for varying lengths of time up to 30 min. The organism is a non-spore former.

Optimum Temperature. There was about equal growth on PGA-phosphate buffer-agar slants when grown at 25°C. and 30°C. Poor growth was obtained at 37°C.

Motility. Cultures 18–24 hr. old, grown in liquid PGA-phosphate buffer or glutamic acid-phosphate buffer mediums were used for motility studies. The organism is non-motile.

Biochemical Characteristics. On a medium consisting of 0.3% beef extract, 1% Difco Bacto peptone, 0.001% brom cresol purple and 1% of each of the following sugars: glucose, maltose, inositol, raffinose, mannitol, galactose, dulcitol, inulin, rhamnose, lactose, arabinose, sucrose, mannose, or xylose, no acid or gas was formed. There was growth in all the tubes but this growth could have been due to the utilization of the amino acid components of the medium (see below).

That the organism can utilize various carbohydrates for growth is apparent from the data of Table II. The basal medium was a *M/5* pH 7.0 phosphate buffer containing 0.05% DL-alanine and Speakman's salts B.² Each sugar was added at a concentration of 0.5%.

The monosaccharides are utilized more readily than the disaccharides with mannitol intermediate between the two groups.

TABLE II
Utilization of Carbohydrates

Compound added	Galvanometer reading
1. Basal	100
2. Basal + DL-alanine	48
3. Basal + DL-alanine + glucose	9
4. Basal + DL-alanine + fructose	8
5. Basal + DL-alanine + arabinose	10
6. Basal + DL-alanine + xylose	10
7. Basal + DL-alanine + mannitol	26
8. Basal + DL-alanine + sucrose	42
9. Basal + DL-alanine + lactose	35
10. Basal + DL-alanine + maltose	34

The miscellaneous biochemical reactions of the organism may be summarized as follows: Nitrates were not reduced to nitrites, litmus milk gave an unchanged reaction, indol was not formed, hydrogen sulfide was not produced, and gelatin was liquified slowly with crateriform to infundibuliform liquefaction.

The organism grows on potato agar to give a thin yellowish growth which later turns brown in color.

Growth on Russell's double sugar agar indicated the organisms to be aerobic. An alkaline reaction was obtained on the surface.

Classification

The organism is similar to *Flavobacterium buccalis*.³ Efforts to obtain a reference culture of this organism for comparison were unsuccessful.

Nutrition of the Organism

Effect of Aeration and Pteroylglutamic Acid concentration. PGA was dissolved at varying concentrations in *M/5* pH 7.0 phosphate buffer containing Speakman's salts B.² Fifty ml. portions were placed in 50 and 250 ml. flasks. After inoculation the former was incubated as a stationary culture while the latter was incubated on a shaking machine. After 3 days at 32°C. the flasks were autoclaved, cooled, adjusted to alkalinity with sodium carbonate, and then warmed to 80° and filtered. Aliquots were

² Speakman's salts B contains 1/100th the normal complement of iron.

³ Subcultures of the organism can be obtained from Dr. B. L. Hutchings, Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

assayed with *L. casei* according to the procedure of Tepley and Elvehjem (2). Pterioic acid is inactive for *L. casei*, so that any residual microbiological activity is due to unchanged pteroylglutamic acid (Table III). The short incubative period of 3 days was chosen to emphasize the differences in the rate of conversion of PGA to pterioic acid in aerated and non-aerated flasks. For maximum formation of pterioic acid a 6-day incubation period is necessary. When the concentration of PGA exceeds 0.05%, aeration greatly favors the degradative reaction. Under optimum conditions, *i.e.*, aeration and a 6 day incubation period, 0.2% PGA is converted essentially quantitatively to pterioic acid. At 0.4% concentration the conversion is in the range of 60-70%.

Effect of pH and Buffer. Acetate buffer at pH 6.0 and 7.0 containing Speakman's salts B² and 0.5% PGA was evidently toxic to the organism. No growth was obtained after 4 days incubation.

TABLE III
*Effect of Aeration and Concentration on the
Bacterial Degradation of Pteroylglutamic Acid*

Concentration of PGA	Conversion to pterioic acid		
	3 days		6 days
	Aerated	Non-aerated	Aerated
<i>mg./ml.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
0.5	96	94	99
1.0	94	83	95
2.0	78	58	94
4.0	65	43	64

In phosphate buffer at pH 6.0 and 7.0 there was good growth and separation of pterioic acid.

Utilization of Amino Acids. The basal medium consisted of M/5 pH 7.0 phosphate buffer and Speakman's salts B.² The addition of 0.05%⁴ of any one of the following amino acids allowed for growth: alanine, valine, leucine, isoleucine, tyrosine, phenylalanine, proline, hydroxyproline, aspartic acid, glutamic acid, and histidine.

Amino acids which were not utilized under the same conditions were: glycine, serine, threonine, cystine, methionine, tryptophan, arginine, lysine, and the peptides *p*-aminobenzoylaspartic acid and *p*-aminobenzoylglutamic acid. The non-utilization of the latter peptide is rather surprising in view of the activity of the compound when incorporated into the PGA molecule.

The amino acids that were utilized satisfied both the carbon and nitrogen requirements of the organism. If a carbon source such as glucose had been present, it is possible that any of the amino acids would have satisfied the nitrogen nutrition of the organism.

Utilization of Inorganic Nitrogen. In the presence of 0.5% glucose as a carbon source, the nitrogen requirements of the organism could be obtained from various ammonium

⁴ The racemic amino acids were used at a concentration of 0.1%.

salts. Such compounds as ammonium sulfate, ammonium nitrate, or ammonium carbonate were readily utilized.

Isolation and Crystallization of Pterioic Acid

A medium containing *M*/5 pH 7.0 phosphate buffer, Speakman's salts B,² and 0.5% PGA was inoculated and then incubated with aeration 6 days at 32°C. The characteristic precipitation of pterioic acid commenced on the second day. At the end of the incubation period the material was autoclaved, cooled, adjusted to pH 8.0 to dissolve any unchanged pteroylglutamic acid, and the precipitate collected on the filter with filter aid. The filter cake, which consisted of crude pterioic acid and filter aid, was slurried in 0.1 *N* potassium hydroxide and filtered. The concentration of pterioic acid in the filtrate should approximate 50 mg./ml. The solution was heated to 80°C. and hot 2 *N* potassium dihydrogen phosphate added to adjust the pH to 7.5. The potassium salt of pterioic acid crystallized on standing overnight at 5°C. This step separates the pterioic acid from any residual pteroylglutamic acid. The precipitate was collected by centrifugation and redissolved in 0.02 *N* potassium hydroxide solution at a concentration of 50 γ/ml. The hot solution was acidified with acetic acid. On cooling pterioic acid crystallized. The precipitate was collected and dried.

Analysis. Calc'd for $C_{10}H_{12}N_6O_8$: C, 53.9; H, 3.12; N, 26.90%. Found: C, 53.7; H, 4.27; N, 26.99%.

The crystallization of pterioic acid both as the potassium salt and as the free acid is practically quantitative. The yield is governed by the completeness of the bacterial degradation.

DISCUSSION

The bacterium is aerobic and, although the fermentation can be carried out in shallow cultures without aeration, the most satisfactory procedure is to incubate the cultures on a shaking machine or for the larger runs sterile air is passed through the medium in the usual manner. In general practice a 0.5% solution of pteroylglutamic acid was incubated the prescribed length of time for a 60–70% conversion into pterioic acid.

The nitrogen requirements of the organism can be satisfied by either inorganic or organic nitrogen. The non-utilization of certain amino acids for growth may be due to their inability to serve as a carbon source for the organism. This point was not further investigated. Of the carbon sources tested, the mono-saccharides are most readily utilized.

When the synthetic approach is not feasible and when pteroylglutamic acid is available as a starting material, the method offers a convenient procedure for the preparation of small quantities of pterioic acid.

SUMMARY

Pteroylglutamic acid in a pH 7.0 phosphate buffer medium containing Speakman's salts B² is converted to pterioic acid by an organism tentatively identified as *Flavobacterium buccalis*.

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Trypsin Inhibitor. VIII. Growth Inhibiting Properties of a Soybean Trypsin Inhibitor ¹

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INTRODUCTION

Ham and Sandstedt (6) observed that extracts of raw soybean oil meal contained a trypsin inhibitor. This led to their assumption (7) that the trypsin inhibitor was responsible for the inferior nutritive value of raw soybean oil meal compared with heated soybean oil meal in which the trypsin inhibitor was inactivated. It was pointed out, however, by Klose, Hill and Fevold (8) that such an assumption had not been proven.

It, therefore, appeared advisable to find out whether or not a purified trypsin inhibitor would have the same effect on growth as the crude acetone precipitate of raw soybean extracts used by Ham, Sandstedt and Mussehl (7). Attempts to prepare the crystalline trypsin inhibitor of Kunitz (9) in sufficient quantities for feeding have been unsuccessful. Instead, the trypsin inhibitor was concentrated from aqueous acid extracts of raw soybean oil meal by salt precipitation and papain digestion of extraneous material. This paper presents data concerning the effect on growth of rats and chicks fed partially purified preparations of a soybean trypsin inhibitor.

PREPARATION OF SOYBEAN TRYPSIN INHIBITOR

In the course of this work, 4 methods for the preparation of soybean trypsin inhibitor were used, yielding progressively a more highly purified preparation of the

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inhibitor. The first preparation, referred to later as the "acetone precipitate," was obtained by mixing raw soybean oil meal² with 10 volumes of 0.05 *N* hydrochloric acid which gave a final pH of 4.2. The suspension was set aside with occasional stirring for 3 hrs. An extract was obtained by filtering through cloth and clarifying in a Sharples steam centrifuge. Acetone was then added to the extract to give a final concentration of 70%. This is essentially the method of Ham, Sandstedt and Mussehl (7). The resulting "acetone precipitate" was removed in the steam centrifuge. By this method, 100 g. raw soybean oil meal yielded 4.5 g. of precipitate which was 6-8 times more active per unit weight in trypsin inhibitor (determined by the method of Borchers *et al.* (2)) than the original meal.

The second preparation, termed the "ammonium sulfate precipitate," was obtained by adding 30 g. ammonium sulfate/100 ml. of the extract obtained as above. The resulting ammonium sulfate precipitate was removed in the steam centrifuge. By this method, 100 g. meal yielded 2.0-2.5 g. of precipitate which was about 15 times more active per unit weight in trypsin inhibitor activity than the original meal.

The third preparation, termed the "sodium sulfate precipitate," was obtained by dissolving the "ammonium sulfate precipitate" in 10 volumes of water and reprecipitating the trypsin inhibitor by adding 25 g. sodium sulfate/100 ml. The precipitate was removed in a high-speed angle centrifuge. This method yielded 1.5-1.75 g. of precipitate from 100 g. original meal and was about 20 times more active in trypsin inhibitor per unit weight than the original meal.

The fourth preparation, termed the "papain precipitate," was obtained by adding to 100 g. meal, a mixture of 240 ml. 95% ethyl alcohol and 45 ml. water at 4°C. This suspension was stirred well and left at room temperature for 30 min. The solvent was removed through cloth and the residue mixed with 500 ml. 0.25 *N* sulfuric acid. The suspension was stirred occasionally for 1 hr. and an extract obtained by filtering through cloth (method of Kunitz (9)). The extract was clarified by passing through a Sharples steam centrifuge. The extract was then adjusted to pH 4.5 by adding sodium hydroxide, 100 mg. papain added/100 ml., covered with toluene, and incubated at 37°C. for 24 hr. A precipitate which separated during the incubation was removed in the steam centrifuge and added to the final precipitate obtained later. The centrifuged solution was adjusted to pH 7.0 by sodium hydroxide, 30 g. ammonium sulfate added /100 ml. and the resulting precipitate removed in the steam centrifuge. This precipitate had no trypsin inhibitor activity and was discarded. The centrifuged solution was then adjusted to pH 2.0 by sulfuric acid and the resulting precipitate removed in the steam centrifuge. The precipitates removed after incubation and after adjusting to pH 2.0 were approximately equal in weight and trypsin inhibitor activity and were combined as the "papain precipitate." By this method, 1.0-1.2 g. of precipitate was obtained from 100 g. meal which had about 50 times as much trypsin inhibitor activity per unit weight as the original meal. The trypsin inhibitor fraction was completely precipitated from aqueous solutions of the "papain precipitate" by 30% ammonium sulfate at pH 2.0 or by 60% ethyl alcohol.

² Solvent processed soybean oil meal prepared with a minimum of heat treatment according to the manufacturer's statement was used in these studies.

FEEDING EXPERIMENTS

The basal ration used in the rat feeding studies is indicated in Table I. The rats were of the Sprague-Dawley strain and were placed on the experimental ration when 21–23 days of age. They were housed in individual screen bottom cages with food and water available *ad libitum*.

TABLE I
Basal Ration for Rat Feeding Experiments

Ingredient	Amount	Ingredient	Amount
	g.		mg.
Soybean oil meal	25.0	Riboflavin	0.5
DL-Methionine	0.6	Thiamine chloride	0.5
Starch plus trypsin inhibitor preparation	51.9	Calcium pantothenate	1.25
Salt mixture ¹	2.0	Niacin	2.5
Cod liver oil	5.0	Pyridoxine hydrochloride	0.125
Lard	15.0		
Choline chloride	0.5		

¹ Salt mixture of Hubbell, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutrition* 14, 273 (1937).

The growth data presented in Table II indicate in each experiment that the more highly purified trypsin inhibitor preparations, "ammonium sulfate precipitate," "sodium sulfate precipitate," or "papain precipitate," were less effective as growth inhibitors than the acetone precipitate. This occurred despite the fact that the trypsin inhibitor content of the ration was higher in each case with the 3 more highly purified trypsin inhibitor preparations than it was in the "acetone precipitate" ration. Similarly, the percentage of growth inhibition as well as significance (*t* value) decreased with increasing activity of the trypsin inhibitor preparations from the "acetone precipitate" to the "papain precipitate." Actually, the average weight gains of the rats receiving either the "sodium sulfate" or "papain precipitate" trypsin inhibitor preparations in their respective rations were not significantly less than that of the rats receiving no trypsin inhibitor in the ration.

The basal ration used in the chick feeding is given in Table III. The ration was prepared in pelleted form as described in a previous publication (Borchers *et al.* (3)). In the chick feeding experiment, 48 one day-

old cockerel White Rock chicks³ were fed the pelleted ration of Lot 362 for the first 4 days. Of these, 32 chicks were selected for best weight gains, distributed among the 4 lots, and placed in individual electri-

TABLE II
Growth Data of Rats Fed Various Trypsin Inhibitor Preparations

Lot	Soybean oil meal	Trypsin inhibitor		Days	No. of rats	Average gain	Per cent of control gain ² ± S.E.	“p” Value ³
		Added	Units/100 g. ration ¹					
255	Heated ⁴	None	0	29	6	48.2	100.0±0.00	
256	Heated	2.4% “Acetone precipitate”	4.8	29	6	40.2	83.4±3.15	5.270 ⁶
258	Heated	1.0% “Ammonium sulfate ppt.”	5.9	29	5	42.5	88.1±4.26	2.793 ⁵
276	Heated	None	0	42	8	73.6	100.0±0.00	
277	Heated	2.0% “Acetone precipitate”	4.7	42	8	59.8	81.3±3.28	5.702 ⁵
278	Heated	0.8% “Sodium sulfate ppt.”	6.1	42	8	67.8	91.8±4.76	1.723
280	Raw	None	8.7	42	8	54.3	74.4±5.83	4.393 ⁶
315	Heated	None	0	35	8	118.0	100.0±0.00	
316	Heated	0.6% “Papain precipitate”	9.6	35	8	111.6	94.7±4.00	1.325
317	Heated	2.0% “Acetone precipitate”	5.9	35	7	92.7	79.0±3.14	6.688 ⁵

¹ Calculated from trypsin inhibitor assay of the soybean oil meal and trypsin inhibitor preparation by the method of Borchers *et al.* (2).

² Per cent of control gain is obtained from litter mate of same sex consuming heated soybean oil meal ration without added trypsin inhibitor.

³ “p” Value according to G. W. Snedecor, Statistical Methods, Iowa State College Press, 1946.

⁴ Heated soybean oil meal was prepared by autoclaving at 15 lbs. pressure for 30 min.

⁵ Significant at the 5% level.

⁶ Significant at the 1% level.

cally-heated compartments. The feeding procedure was that of Ackerson, Blish and Mussehl (1). All chicks were kept at approximately the same total feed consumption through the first 3 weeks. Lots 364 and

³ We are indebted to Mr. I. L. Williams of the Department of Poultry Husbandry for careful sexing of the chicks.

TABLE III
Basal Ration for Chick Feeding Experiment

Ingredient	Parts by weight	Ingredient	Parts by weight
Yellow corn meal	24	Meat scraps	6
Corn gluten meal	5	Salt mix No. 45 ¹	3
Wheat shorts	10	Vitamin D carrier ²	1
Wheat bran	10	Dextrin	10
Dehydrated alfalfa meal	5	Gum tragacanth	2
Soybean oil meal	23	Talc	1

¹ Limestone 60 lbs., iodized NaCl 30 lbs., manganese sulfate 12 oz.

² 200 A.O.A.C. chick units/g.

365 then began to lag behind in food consumption so that it seemed advisable to allow Lots 362 and 363 to proceed at a more rapid rate. Each chick of Lots 362 and 363 consumed 800 g. ration by the age of 40-42 days, Lots 364 and 365 consumed 700 g. in the same period. The growth data, presented in Table IV, indicate that the "papain precipitate" trypsin inhibitor preparation had no growth inhibitor activity for the chick. The growth inhibition of the "acetone precipitate" in the case of the chicks was greater than that found with rats.

TABLE IV
Growth Data of Chicks Fed Various Trypsin Inhibitor Preparations

Lot	Soybean oil meal	Trypsin inhibitor		Days	No. of chicks	Food consumption	Average gain g. \pm S.E.	Efficiency ³
		Added	Units/100 g. ration ¹					
362	Heated ²	None	0	42	8	800	349 \pm 5.5	43.6
363	Heated	0.5% "Papain precipitate"	8.0	42	8	800	356 \pm 3.9	44.5
364	Heated	2.0% "acetone precipitate"	6.0	42	8	700	248 \pm 5.6	35.4
365	Raw	None	8.1	42	8	700	240 \pm 6.7	34.3

¹ Calculated from trypsin inhibitor assay of the soybean oil meal and trypsin inhibitor preparations by the method of Borchers *et al.* (2)

² Heated soybean oil meal was prepared by autoclaving at 15 lbs. pressure for 30 min.

³ Gain/food consumption \times 100.

DISCUSSION

Bowman has obtained 3 trypsin inhibitors from soybeans (5). The trypsin inhibitor preparation termed the "papain precipitate" in this paper appears to be identical with the trypsin inhibitor of Kunitz (9) and with the alcohol-insoluble factor of Bowman (4), since it is precipitated with 30% ammonium sulfate or with 60% ethyl alcohol. The data presented in this paper establish, hence, that only one of the known soybean trypsin inhibitors lacks growth inhibitor activity.

SUMMARY

A partially purified trypsin inhibitor was prepared from raw soybean oil meal by acid extraction, simultaneous papain digestion and partial precipitation at pH 4.5, and further precipitation with 30% ammonium sulfate at pH 2.0. This trypsin inhibitor preparation had no significant effect on the growth of rats or chicks.

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Lactation in Rats on Well Fortified All-Plant Rations

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INTRODUCTION

Van Landingham and Lyon (1) have described as inadequate for good lactation and growth of young rats a basal diet made up of 20% of soybean meal and grains, and containing, in addition, bone meal, a small amount (2%) of alfalfa, fat-soluble vitamins, and riboflavin. It is furthermore stated that addition of 2% dried pig's liver resulted in adequate lactation, while this was not the case for supplements of 5% of additional alfalfa meal or 5% of yeast, although the latter two supplements did improve the condition. They quite rightly attribute the failure to "either a lack of sufficient milk or a lack of some factor or factors in the milk." The present paper presents evidence bearing on these alternatives.

EXPERIMENTAL

The diet we have used (2) is described as a well fortified all-plant ration. The main protein source is either cottonseed meal or soybean meal. It approximates the equivalent of Van Landingham's basal diet plus both his partially successful supplements yeast and alfalfa.

When dams are put on such a diet at the time of impregnation, the young grow normally like stock diet animals for 2.5-3 weeks of age. By the time they are weaned (at 4 weeks) there is, however, a noticeable growth retardation, and this continues unless a supplement of zoopherin (2) is given. During the preweaning period, deaths were as rare as among stock diet animals, but shortly after weaning at 4 weeks considerable mortality was encountered.

Numerous findings in the somewhat scattered literature indicate that lactation is adversely affected, as is growth, by almost all deficiencies. Cerecedo and coworkers (3)

failed to find any specific lactation-inhibiting deficiencies, and when, in purified diets, all known factors including folacin were given, lactation was good. Nelson and Evans (4) produced evidence to show that even with folacin in the purified diets another factor which occurs in liver was needed to bring lactation quite up to stock diet levels. Both these investigators judged success of lactation not only by the growth of the offspring but also by the body weight behavior of the lactating dam.

Table I gives the mean data for 50 dams and offspring on diet Pr 60 when the deficient regime was started at the time of impregnation. The weights of the mother rats behave like those on stock diet and the young grow normally to nearly 3 weeks of age.

TABLE I
*Weights of Dams and Offspring with Short-Term Zoopherin Deficiency;
Cottonseed and Soy Diets*

N ^a	Diet	Weight of dam Days postpartum					Mean weight of offspring ^b Days postpartum				
		0	7	14	21	28	0	7	14	21	28
50	Pr 60	280	295	296	296	284	5.3	13	25	38	55
4	Soy 60	270	262	264	258	247	5	11	19	29	43
7 ^c	Stock	276	287	293	289	274	5.4	14	27	42	65

^a N = number of litters.

^b All litters reduced to 6 within 4 days after birth. Due to subsequent deaths, 1 of the Soy 60 litters consisted of only 4 rats, another 5. The mortality in the Pr 60 litters was very small—no greater than in stock litters.

^c The 7 litters were from the same 4 dams that are listed under "Soy 60." They had litters on stock diet both before and after the experiments with soy.

In an earlier paper (2) we have also described experiments with longer deficiency periods of many months duration. Under these conditions some early deaths were recorded, and we indulged in the offhand speculation that this might have been due to faulty lactation. Since then, problems relating to lactation have come up more acutely and we are giving in Table II a comparison of Pr 60 litters produced after deficiency of longer standing, with normal litters produced by the same dam on stock diet or a plant ration fortified with 15% of crude casein (Diet Pr P). Here also, there are no indications that zoopherin deficiency produces any noticeable lactation failure. Even with the longer depletion time, there is no reason for attributing these early deaths to

TABLE II
Weights of Dams and Offspring when the Zoophorin Deficiency is of Long Standing

Rat	Diet	Weight of dam Days postpartum				Mean weight of offspring Days postpartum					Remarks	
		0	7	14	21	28	0	7	14	21		28

Series A												
39264	Pr 60	216	235	248	246	238	4.7(9)	9(6)	22(5)	34(3)	49(3)	1 dead 0 d.; 2 dead 4 d.; 1 dead 14 d.; 2 dead 18 d.
	Pr P	252	266	268	264	254	5.7(10)	15(6)	28(6)	40(6)	65(6)	No deaths
39275	Pr 60	228	244	264	253	240	5.5(9)	11(6)	22(6)	34(6)	48(6)	1 dead 0 d.; 1 dead 3 d.
	Pr P	282	294	298	295	290	5.7(7)	15(6)	24(6)	40(6)	62(6)	No deaths
39276	Pr 60	229	240	274	278	273	4.6(12)	8(6)	12(6)	23(5)	39(5)	2 dead 3 d.; 1 dead 21 d.
	Pr P	290	308	316	322	312	5.4(13)	11(5)	21(5)	43(5)	64(4)	1 dead 4 d.; 1 dead 7 d.; 1 dead 24 d.
39426	Pr 60	214	211	226	228	218	5.7(11)	9(3)	21(2)	37(2)	52(2)	1 dead 0 d.; 2 dead 3 d.; 3 dead 4 d.; 1 dead 11 d.
Series B												
39246	Pr P	280	298	312	300	290	4.6(14)	13(6)	29(6)	48(6)	75(6)	No deaths
	Pr 60	304	326	324	323	294	5.0(13)	14(6)	27(6)	35(6)	46(6)	No deaths
	Pr 60	290	302	310	293	289	5.4(14)	7(6)	11(5)	20(5)	25(5)	1 dead 7 d.; 1 dead 10 d.
39448	Stock	244	254	268	270	262	5.0(12)	15(6)	31(6)	45(6)	72(6)	No deaths
	Pr 60	248	278	284	278	268	5.9(12)	17(6)	29(6)	42(6)	55(6)	No deaths
	Pr 60	248	268	270	288	274	4.5(10)	7(4)	13(3)	25(3)	30(3)	1 dead 0 d.; 4 dead 3 d.; 1 dead 14 d.

Series A—Eight females on Pr 60 from 28 days of age; first litter on Pr 60, second on Pr P (normal diet). In the case of 4 of the 8 females the litter died so young that no lactation weights could be recorded. The full growth of the dams is given in right hand of Fig. 2 (ref. 2).

Series B—Ten females which were raised on normal diets and had a litter on a normal diet. They were then transferred to Pr 60 and had 2 litters. The first litters on Pr 60 had no deaths and growth was normal to nearly 3 weeks. Of the second litters on Pr 60, 8 died within 3-4 days after birth; the table refers only to the rats with second litters surviving.

lactation failure rather than to an exacerbated deficiency state in the young themselves. The obvious conclusion is that, in the presence of satisfactory amounts of milk, its low zoopherin content is responsible for the result.

Table I also presents the somewhat different behavior of animals on diets which contained 60% of soybean meal instead of the cottonseed meal. Judging by the weight of the dam, lactation appears to be slightly deficient. In these animals it was noted that, although the postweaning growth was, like that on diet Pr 60, subject to the same beneficial effects of liver supplements, there were some preweaning deaths and fewer acute deaths after the animals were weaned. This behavior of poorer growth leading to less acute symptoms is reminiscent of the non-appearance of kidney hemorrhage in choline deficiency experiments unless the previous growth is good. As stated previously (2), postweaning mortality is variable and seems to be related to the state of the particular dam. Whether this is dependent on storage related to composition of the stock diet, which, after all, may vary a little from time to time, or to intestinal synthesis, we are not ready to say. We have recently not lost nearly as many young animals as was recorded earlier, when, at times, the mortality approached 50%. Fortunately for testing purposes the body weights were not subject to so much variability as the survival.

DISCUSSION

We have stated that, in order to study zoopherin deficiency, it is essential that all other factors be provided in the diet in ample amounts. With a diet as well fortified with various natural foods as diet Pr 60, this is more likely to be the case than with more purified diets.

There are two outstanding difficulties in interpreting the role of dietary factors in lactation. There is uncertainty as to the extent of the presence of what are now known factors or such unknowns as were discussed in connection with seed meals and alfalfa (5). A large doubt also frequently exists as to the purity of the casein. The work of Cary and Hartman (6) on exhaustive extraction with hot alcohol is of basic clarifying value in the development of the whole subject under discussion. All treatment of casein will become increasingly important as further work with purified diets progresses.

The question has been raised whether there may be a basic flaw in using oil seed meals in work of this type for fear of toxic action. The evidence for a toxic factor in

soybean meal (7, see also discussion by duVigneaud, 8, p. 203) has not been and possibly need not be taken very seriously. Raw cottonseed, however, does contain a substance, gossypol, which needs to be considered in this respect. In our previous report (2) we stated that the preparation we used routinely (Proflo) was made under very well controlled conditions of heat and moisture treatment with thorough inactivation of the gossypol, and that the results with an exhaustively ether-extracted cottonseed were no different. Since then we have had the opportunity of using also the "flotation process" cottonseed meal of Boatner *et al.* (9), from which the so-called pigment glands which contain the gossypol have been removed as such. This did not differ from the ether-extracted or heat-inactivated preparations in any of the properties associated with diet Pr 60.

Is there a chance that liver counteracts a possibly residual gossypol effect? We have reported (10) that small doses of gossypol can reduce food intake without any other obvious or lasting results. Recent experiments on supplying extra B factors (thiamine, riboflavin, pyridoxin, calcium pantothenate, niacin) to such animals will slightly reduce the body weight deficit which is due to the lowered food consumption. There is, however, no additional effect of 1:20 liver powder when given in amounts which lead to growth resumption in zoopherin-deficiency experiments.

As a matter of fact, for long time experiments a well processed cottonseed meal is a useful diet component. It possesses an excellent vitamin B complement except for a low niacin value and, when fed at levels of total nitrogen required by young rats, reveals in growth experiments no amino acid deficiencies (11). The outstanding property which distinguishes it from some other seed meals and grains is its stability, in the sense of absence of rancidity even when kept for long periods of time. The troublesome disappearance of vitamin A value which will occur in many diets has, during a number of years of experience, never been observed in cottonseed meal diets kept at room temperature.

SUMMARY

When female rats are carried through a pregnancy and lactation on a well-fortified plant ration based on cottonseed flour, lactation, judged both by the weight of the dam and the offspring appears to be normal. If soybean meal is substituted for the cottonseed meal, lactation is depressed.

If females are maintained on the cottonseed diet for many months and then bred, there is high mortality and poor preweaning growth in the offspring, but the weight gain of the dam during the lactation period is normal. This is interpreted to mean that there is a normal quantity of milk produced, which is deficient in composition.

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Inositol, an Active Constituent of Pancreatic (α) Amylase

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INTRODUCTION

Williams, Schlenk and Eppright (1) found that a highly purified preparation of pancreatic amylase contained 4.1 mg. inositol/g. and concluded that inositol appeared to be a constituent of pancreatic amylase.

Meyer, Fischer and Bernfeld (2) found that pancreatic amylase could be deactivated by dialysis, giving a heat-labile component of protein nature and a dialyzable thermostable component. Recombination of the dialyzed enzyme with its dialyzate did not restore the activity; in fact, the dialyzed enzyme inactivated an active preparation. This inhibitory effect of the dialyzed enzyme could be prevented by the addition of the dialyzate (coenzyme). They concluded that the protein portion of the enzyme was changed on dialysis in such a way that it could still recombine with the coenzyme but could not regain its enzymatic activity.

Kirkwood and Phillips (3) demonstrated that the γ isomer (m. p. 112°C.) of 1,2,3,4,5,6-hexachlorocyclohexane inhibited the growth of the Gebrüder Mayer strain of *Saccharomyces cerevisiae*. This inhibition was found to be prevented by the addition of *D*-inositol. Buston, Jacobs and Goldstein (4) found that *Nematospora gossypii* was also reversibly inhibited by γ -hexachlorocyclohexane. This organism requires an exogenous supply of inositol for optimum growth.

In view of these facts, it seemed that γ -hexachlorocyclohexane might be used to gain evidence with respect to the function of inositol as an active constituent of pancreatic amylase.

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EXPERIMENTAL

Inositol Assay

The method of Williams *et al.* (5) was used for all inositol assays.

Amylase Assay

The substrate for the enzyme was prepared by dispersing in a boiling solution of 0.02 *M* sodium chloride and 0.01 *M* phosphate buffer, pH 7.0–7.1, sufficient soluble starch (according to Lintner, Eimer and Amend) to make a 2% dispersion (6). As soon as the starch was added, the flask was plugged with cotton and the mixture allowed to cool. This procedure produced a mixture sufficiently free of microorganisms so that it maintained a low reducing sugar content (less than 2% of theoretical) for 3–5 days. This low level was maintained when only a sufficient amount of substrate was poured out of the flamed flask each time the solution was used. Making up small quantities of the starch substrate every 3 days was found to be more convenient than sterilizing larger amounts and taking elaborate precautions to maintain this sterility over a longer period of time.

Portions (5 ml. or 25 ml.) of this substrate were put in small bottles in a shaker and brought to 40°C. in a constant temperature water bath. The Schultz, Atkin and Frey fermentometer (11) (Am. Instrument Co.) was found to be a convenient device for this purpose. Samples (0.2 ml. or 1 ml.) of the material to be assayed were added to the various bottles in a definite order and the bottles stoppered. A starch blank was included in each run.

After 30 min., the shaker was stopped and 2 ml. of the reacted mixture were removed from the bottles in the same order that the samples had been added. These 2 ml. portions were added to 2 ml. of Benedict's (7) sugar reagent contained in 25 ml. Folin-Wu sugar tubes. A standard solution of maltose (2 ml. of 0.5 mg./ml.) was also included in each series of sugar determinations.

The analyses for reducing sugar were performed according to the method of Benedict (7). The color developing in the samples was compared with the color developed in the maltose standard in a Klett-Summerson photoelectric colorimeter, or in a Dubosq visual colorimeter.

The total activity of a particular sample is expressed as the number of milligrams of maltose produced under the described conditions.

Materials

The γ isomer of hexachlorocyclohexane was obtained by separating and purifying it from Eastman benzene hexachloride (practical) according to the method of Slade (8). Crystals melting 112–112.7°C. were obtained.

A portion of the purified pancreatic amylase originally supplied to this laboratory (1) by Dr. Caldwell in 1943 was still at hand. This material originally had an activity of 11,000 and an inositol content (1) of 4.1 mg./g. Under the conditions of the present assay, it still had an activity of 9000 and a reassay for inositol showed it to have the same inositol content.

A preliminary experiment with this preparation showed that it could be deactivated with γ -hexachlorocyclohexane. For continuation of the study additional quantities of highly purified materials were prepared.

Using pancreatin (Merck) as the starting material, 3 batches were prepared following the procedure of Sherman *et al.* (6).

A β -amylase concentrate was also prepared from "clarase" by a procedure similar to that of Cladwell *et al.* (9) with the following changes:

1. The clarase was held for 24 hr. at 0°C. at pH 3.4 to destroy the α -amylase (10).
2. Between each of the ammonium sulfate fractionations a water solution of the enzyme was passed over acetate-treated amberlite IR-4, instead of being dialyzed to remove the sulfate.

The activity of β -amylase was determined by measuring the reducing sugar produced in 30 min. at 40°C. from 1% soluble potato starch (according to Lintner) adjusted to 0.05 *M* sodium chloride and to 0.01 *M* acetate at pH 5.0 (9). The clarase had an original activity of 350 and an inositol content of 2.1 mg./g. The most active concentrate obtained had originally an activity of 3500 and an inositol content of 0.5 mg./g. The activity at the time of the inhibition study had decreased to 2500 and the inositol content remained the same.

RESULTS

It was apparent from preliminary experiments that inhibition by γ -hexachlorocyclohexane does not take place unless there is a considerable period of contact. Presumably, the replacement of inositol in the enzyme by the inhibitor takes place slowly. To determine the time required for inhibition to develop under a given set of conditions, the following experiment was run: 10 ml. of 120 γ /ml. solution of γ -hexachlorocyclohexane in dioxane were added to 10 ml. of a solution of the enzyme of activity 4 units/ml. in 0.02 *M* phosphate buffer, pH 7.0 (dioxane was used as the solvent for the inhibitor because it had no deleterious effect on the enzyme, *i.e.* it did not inactivate the enzyme or precipitate it up to 50% concentration). The resulting mixture was 0.01 *M* in phosphate, contained 60 γ of inhibitor/ml., and had an activity of 2 units/ml. This mixture was held at 9°C. in the refrigerator. Samples (1 ml.) were removed and assayed after 0, 4, 8, 15, and 14 hr. A portion (0.2 ml.) of the sample was added to 5 ml. of the starch substrate immediately upon removal, and assay conducted as described for the assay of pancreatic amylase. A control containing 10 ml. of dioxane but no inhibitor was run at the same time. The results are

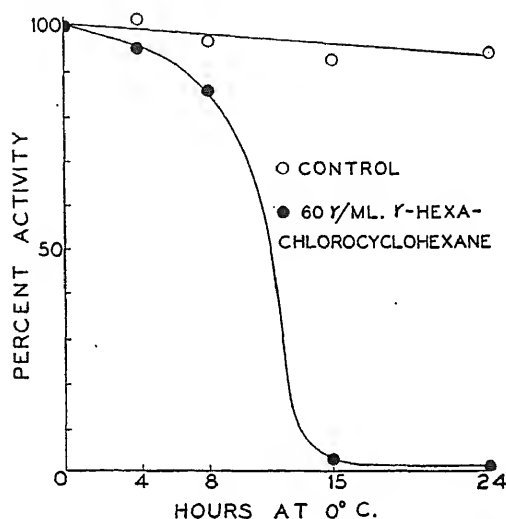


FIG. 1. Inactivation of pancreatic amylase by γ -hexachlorocyclohexane. \circ , Control, 10 ml. of enzyme solution (4 units/ml.) plus 10 ml. dioxane; \bullet , 10 ml. of enzyme solution (4 units/ml.) plus 10 ml. of dioxane solution of γ -hexachlorocyclohexane (120 γ /ml.).

shown in Fig. 1. Fifteen hours at 0°C. was selected as the standard time of contact of the inhibitor with the enzyme in subsequent experiments.

To determine the molar inhibition ratio, 10 ml. of inhibitor solutions containing graded amounts of γ -hexachlorocyclohexane were added to a series of 10 ml. of enzyme solutions each having an activity of 4 units/ml. and an inositol content of 1.4 γ /ml. This series of mixtures was held at 0°C. for 15 hr. after this time, 1 ml. samples were withdrawn and assayed. Table II shows proportions of inhibitor to inositol and the results obtained.

The molar inhibition ratio is therefore 50. This is in comparison with

TABLE I
Result of Concentration of Pancreatic Amylase

Batch	Final activity	Inositol content mg./g.
I	5,280	3.0
II	9,000	3.8
III	10,000	3.9

Batches II and III were combined and used in the inhibition studies.

the molar inhibition ratio of 30 found by Lockwood and Phillips (3) with yeast.

To determine whether this inhibition can be prevented by inositol, 10 ml. of a dioxane solution containing 120 γ /ml. of the inhibitor were added to a series of 10 ml. of enzyme solution having an activity of 4 units/ml. and an inositol content of 1.4 γ /ml. Graded amounts of inosi-

TABLE II
*Effect of γ -Hexachlorocyclohexane when Various
Molar Ratios of Inhibitor to Inositol are Used*

Amount of inhibitor /20 ml. γ	Resulting molar ratio	Activity remaining per cent
0	—	100
24	1	100
120	5	91
240	10	80
480	20	67
720	30	35
960	40	15
1200	50	1
1800	75	1
2400	100	1

TABLE III
*Prevention of Amylase Inhibition by
 γ -Hexachlorocyclohexane by Inositol*

Amount of inositol /ml. γ	Resulting molar ratio	Activity per cent
0	50	1
2	42	8.5
5	33	31
10	22	61
30	10	90
50	6.5	99

tol were added in 1 ml. portions to each solution. After 15 hr. at 0°C., 1 ml. aliquots were assayed for enzyme activity. Table III shows the amounts of inositol per ml. and the resulting activity.

Effect of Inhibitor on β -Amylase

Ten mg. of the 2500 unit material from clarase was dissolved in 1 liter of water and adjusted to pH 5.0 with 0.05 *M* phosphate buffer. This

solution had an activity of 25 units and an inositol content of 0.005 γ /ml. A 10 ml. portion of this solution was mixed with 1 ml. of γ -hexachlorocyclohexane solution containing 11 γ /ml. This is a molar ratio of 120. After standing in the refrigerator for 24 hr., a 1 ml. sample was assayed. A control sample containing 1 ml. of dioxane was treated in the same manner. The mixture containing the inhibitor retained 92% of its activity while the control retained 95% of its activity.

DISCUSSION

The fact that the inhibition of pancreatic amylase by γ -hexachlorocyclohexane was competitively prevented by the addition of inositol is evidence that inositol is an active constituent of α -amylase. If this is so, a biochemical function of inositol has been found.

The fact that a highly concentrated preparation of β -amylase contained little inositol and that the enzyme was not appreciably inhibited by γ -hexachlorocyclohexane indicates that inositol is not an active constituent.

SUMMARY

γ -Hexachlorocyclohexane inhibited the activity of a purified preparation of pancreatic amylase. This inhibition was competitively prevented by inositol.

Contact between the inhibitor and the enzyme must be maintained for at least 15 hr. at 0°C. in the proportion of 50 moles of inhibitor to 1 mole of inositol to inactivate the enzyme completely.

These data indicate that inositol is an active constituent of α -amylase.

Parallel experiments indicate that inositol is not an active constituent of β -amylase.

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LETTERS TO THE EDITORS

Incorporation of Carbon-Labeled Formic Acid and Carbon Dioxide into Hypoxanthine in Pigeon Liver Homogenates¹

The experiments reported here demonstrate the synthesis of a purine in essentially cell-free pigeon liver homogenates by the incorporation of labeled formate and bicarbonate into hypoxanthine. Results of some of these studies are shown in the accompanying table.

Örström *et al.* (1) had previously observed an increased formation of hypoxanthine in pigeon liver slices using glutamine and oxaloacetic acid substrates, but they were unable to demonstrate the reaction in a cell-free system. Recently it has been shown by Sonne and coworkers (2) that *in vivo* HC^{13}OOH is incorporated into carbons 2 and 8 and that C^{13}O_2 is fixed into carbon 6 of uric acid in the pigeon.

The experiments were carried out as indicated in the table legend. Non-radioactive hypoxanthine was added as a carrier at the end of the experiment. The hypoxanthine was isolated first as the mercury salt, then as the silver nitrate salt, the latter being recrystallized from hot nitric acid 3 times, and finally the free hypoxanthine was crystallized from water 3 times. Successive recrystallizations from water showed the same specific activity. The purity of the hypoxanthine was established by its ultra-violet absorption spectrum and its quantitative conversion to uric acid by xanthine oxidase using the techniques of Kalekar (3).

It will be seen that the net formation of hypoxanthine as measured spectrophotometrically with xanthine oxidase (3) may be considerably greater than that calculated from the C^{14} formate incorporated into the purine. This difference could arise from concomitant breakdown of nucleic acid fragments, or by the synthesis of hypoxanthine from unlabeled endogenous precursors (2). The data (Exp. 2a and b) suggest that purine synthesis as determined by C^{14} incorporation may proceed anaerobically at a much slower rate than aerobically, while the total formation of hypoxanthine does not vary significantly. In a number of

¹ Aided by a grant from The National Foundation for Infantile Paralysis.

TABLE I

Expt.	Reaction time ^a	Gas phase	Hypoxanthine		C ¹⁴ cpd. added			Incorporation of C ¹⁴ compound into hypoxanthine	
			Initial	Final					
1a	min. 67	O ₂ -CO ₂	μM 0.51	μM 2.94	cpd. HCOOH	ct. 5720	μM 1.28	ct. 1760	μM^b 0.39
b	0	O ₂ -CO ₂	0.51	—	HCOOH	5720	1.28	0	0
2a	60	O ₂ -CO ₂	0.14	2.68	HCOOH	5720	1.28	3680	0.82
b	60	N ₂ -CO ₂	0.14	2.46	HCOOH	5720	1.28	945	0.21
3a	30	O ₂ -CO ₂	0.14	1.19	HCOOH	5720	1.28	2100	0.47
b ^c	30	O ₂ -CO ₂	0.14	0.78	HCOOH	5720	1.28	2530	0.57
4a	31	O ₂	0.32	1.37	CO ₂	43900	79.7	413	0.75

^a No additions, see legend.

^b Endogenous formate or bicarbonate formation neglected in calculation.

Values are in terms of total vessel contents. Radioactivity is expressed in ct./min.; 1.75×10^5 ct./min. is approximately 1 microcurie. Tissue homogenized 10 min. in Potter-Elvehjem apparatus with 3 parts of the internal salt solution of Lipmann with 0.03 M phosphate buffer, pH 7.4 (4). Reaction vessels (except 4a) contained 2 ml. of homogenate, 0.2 ml. 0.4 M KHCO₃, C¹⁴ sodium formate as shown, KOH in center well except 2b, gas phase 95% O₂-5% CO₂, or 95% N₂-5% CO₂ as given. In 1a, b, and 2a, b, and 3a, the following additional substances were added in final concentrations: 1.2×10^{-5} M cytochrome c (except 2b), 0.01 M K pyruvate, 0.01 M glutamine, 0.005 M K fumarate. Vessels 3b and 4a did not have these additions. Vessel 4a contained 2 ml. of homogenate, 1 ml. 0.080 M KHC¹⁴O₃, 0.15 ml. 0.1 M KH₂PO₄; gas phase, O₂; no KOH in center well. Final volumes: Expts. 1, 2, and 3-5 ml., Expt. 4-3.15 ml. Reaction temperature: 38.1°C. After incubation, 5-10 mg. of hypoxanthine were added as a carrier.

experiments (compare 3a and b) addition of substances which might be expected to enhance the incorporation of C¹⁴ formate into the purine actually appeared to decrease the incorporation. Attempts to deplete the endogenous substrate to facilitate the study of the effect of added substrate have resulted in much less active systems. Of interest is the observation not shown in the table that 0.001 M adenosine triphosphate consistently resulted in less incorporation of radioactivity into the purine. This probably is not due to the observed breakdown of adenosine triphosphate to hypoxanthine by this system, since addition of hypoxanthine to 0.008 M concentration before incubation did not significantly alter the C¹⁴ incorporation. The effect of related com-

pounds, such as muscle adenylic acid or adenosine, has not been tested on this system. Exp. 4 illustrates the fixation of C^{14} bicarbonate into hypoxanthine.

On a weight basis, pigeon liver mince did not bring about more incorporation of C^{14} formate into the purine than did the homogenate, indicating that, in the latter, it was not the presence of a few cells that was responsible for the reaction.

This system is being employed to study the intermediate reactions involved in the synthesis of hypoxanthine and to follow its metabolism.

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The Coenzyme of Phosphoglucomutase

Estimation of the coenzyme of yeast phosphoglucomutase (1) has shown that its concentration in yeast increases 100-fold under the same conditions which lead to an accumulation of fructose diphosphate (2). The separation of these two substances by fractionation has not been possible, but treatment with alkali until the Seliwanoff reaction becomes negative does not affect the coenzyme.

The most highly purified preparations contain acid labile and acid stable phosphate in a 1:1 ratio. On mild acid hydrolysis (7 min. at 100°C. in 1 *N* acid) the activity is destroyed, and one equivalent of aldose is liberated (hypoiodite titration).

Active preparations have been obtained by treatment of 1, 6-di-bromotriacetylglucose with silver phosphate, but the yield was so low that the reaction was useless for preparative purposes.

The coenzyme can be synthesised by an enzymatic reaction on incubation of adenosinetriphosphate, glucose-1-phosphate, and a partially purified yeast enzyme (Table I). Replacement of glucose-1-phosphate

TABLE I

Synthesis of Cophosphoglucomutase from Glucose-1-Phosphate and ATP

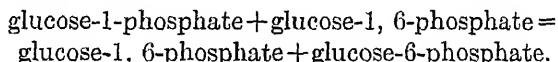
Incubation 10 min. at 30°C. of: 0.05 ml. partially purified enzyme + 0.04 M Mg^{++} + 0.01 M phosphate buffer of pH 7.4 + 3.8 μM ATP + 10 μM glucose-1-phosphate. Total volume 0.3 ml.

	Coenzyme formed ^a
Enzyme + ATP + glucose-1-phosphate	1.05
Enzyme + ATP	0.07
Enzyme + glucose-1-phosphate	0.03

^a Measured on a 0.02 ml. aliquot of the deproteinized reaction mixture by adding 1.5 μM glucose-1-phosphate, 0.005 M Mg^{++} and 0.02 ml. yeast phosphoglucomutase. After 10 min. at 30°C., glucose-6-phosphate was estimated by its reducing power. Results in μM of glucose-6-phosphate. (The rate of formation of glucose-6-phosphate is proportional to the amount of coenzyme.)

by glucose, fructose, Embden ester, or a mixture of the fructose-1- and 6-phosphates gave a negligible yield of coenzyme.

While positive identification will have to await isolation of larger amounts of the substance, these facts are consistent with our working hypothesis that the coenzyme is glucose diphosphate and that its mechanism of action is:



The action of this coenzyme is not limited to yeast phosphoglucomutase since the rat kidney and heart enzymes are also activated.

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Transformation of Ovalbumin into Plakalbumin

In a previous note (1) experiments were reported in which it was shown that ovalbumin, when acted upon by an enzyme from *Bacillus subtilis*, is transformed into another protein which we have termed

plakalbumin (from Greek $\pi\lambda\alpha\kappa$: plate) because it crystallizes in rectangular plates. During the past year this process of transformation has been studied in some detail with the following general results:

The enzymatic process involves a scission of peptide bonds (and possibly other bonds) and a liberation of nonprotein nitrogen. When about 1.2–1.4% nonprotein N (~ 6 –7 atoms of nitrogen per protein molecule) is set free, plakalbumin can be isolated in crystalline form and in a yield of between 95 and 96% of the theoretical. Prolonged action of the enzyme results in a slow breakdown of plakalbumin, a liberation of more nonprotein N, and a decrease in crystal yield. We

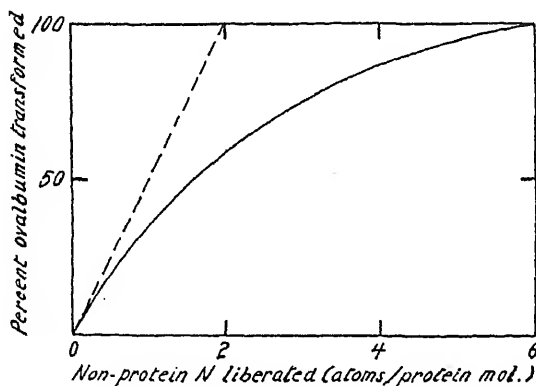
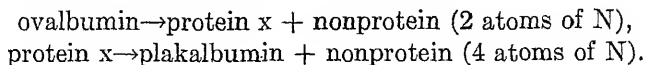


FIG. 1. Relation between the formations of nonprotein and of plakalbumin.

shall refer to the latter process as the non-specific reaction as distinct from the specific reaction leading to the maximum yield of plakalbumin.

Independent of the pH of the reaction mixture, and of the purity and concentration of the enzyme, the same curve is found when plotting "per cent ovalbumin transformed" against "nonprotein N liberated" in the specific reaction (see Fig. 1). Since this curve is not a straight line, it must be concluded that plakalbumin formation proceeds in more than one step, the simplest assumption being:



(Compare slope of curve at zero point.)

Five per cent ovalbumin solutions containing up to 0.05% *subtilis* enzyme were digested at pH 5-8 and 20°C. The specific reaction lasted from 3 to 30 hr. Nonprotein N was determined by precipitation with trichloroacetic acid (10%) and the disappearance of ovalbumin was followed by titration with 95% ammonium sulphate solution, making use of the observation that the turbidity point is displaced toward higher ammonium sulphate concentration when ovalbumin is replaced by plakalbumin (see (1)). The titration values were converted to "per cent ovalbumin" by means of standard curves obtained by titration of known mixtures of the two proteins.

Some elements of the chemistry of the reaction will appear from the following table.

TABLE I

Property substances or groups	Ovalbumin	Plakalbumin	Ultrafiltrate from re- action mix- ture	Investigator
Molecular weight Ultracentrifugal Osmometric	45,000	43,700 44,500-45,000		K. O. Pedersen A. V. Güntelberg
N, %	15.6	15.6		Authors
P, mg./g. N	8.1	8.0	0	Authors
SH, % cysteine	1.3	1.3	0	E. Fredericq
Mannose, %	2.2	2.2		E. Fredericq
Sugar (Molisch)			0	H. A. Lillevik
NH ₃ (Nessler)			0	H. A. Lillevik
Tyrosine (Millon)			0	H. A. Lillevik
Tryptophan (Komm and Böhringer)			0	Authors
Filter paper chromatog- raphy (2):				
Amino acids			0	Authors
Amino acids in peptides after hydrolysis:				
Alanine			+	Authors
Glycine			+	Authors
Valine			+	Authors
Glutamic acid			+	Authors
Aspartic acid			(+)	Authors

A quantitative analysis of a concentrated ultrafiltrate obtained during the latter part of the specific reaction gave the following result:

NH ₂ -groups	2.0 per 6 nitrogen atoms
COOH-groups	3.6 per 6 nitrogen atoms
peptide-bound nitrogen	4.0 per 6 nitrogen atoms

This is in fair agreement with the assumption that 2 peptides, one dipeptide, and one tetrapeptide (or 2 tripeptides), are formed, containing 2 molecules of monoaminodicarboxylic acid. Unpublished electrophoretic data of Perlmann (3) are in agreement with this conception.

As regards the physical properties of plakalbumin we may refer to a note by Perlmann (4) in which the question of the homogeneity of plakalbumin in the electric field has been taken up and a comparison with previous electrophoretic studies by MacPherson, Moore and Longworth (5) is made. At the present moment we feel unable to discuss the different phenomena observed, but we should like to call attention to the following two points:

1. Due to the complexity of the "specific" reaction and to the existence of a nonspecific breakdown the possibility exists that plakalbumin is contaminated with related proteins that are able to form mixed crystals with it. In this connection it should be mentioned that, while dissolved plakalbumin is perfectly stable at pH 4.8-5 where both the specific and the nonspecific reaction are greatly repressed, slow break-down is observed at pH 6.4 (20°C.) even after repeated recrystallizations and dialysis. This indicates that the enzyme remains active for a long time and warns against long-time experiments in the neighborhood of the pH optimum of the enzyme (about 8).

2. While we have found that no phosphoric acid is split off from ovalbumin in short-time enzymatic experiments (see Table I) we have definite evidence that the presence of phosphorus in plakalbumin is unessential for its crystallization in plates. A 22 year old, spontaneously infected ovalbumin solution yielded magnificent plate crystals that contained less than 0.05 mg. P/g. N. This observation, which calls to mind the phosphorus-poor ovalbumins (6) of Mâcheboeuf, Sørensen and Sørensen, seems to us of considerable interest in view of its possible importance in the identification of the different components separated in the electric field.

Details of the experiments mentioned in the present note will be published shortly in the *Compt. rend. trav. lab. Carlsberg*.

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Book Reviews

The Chemical Constitution of Natural Fats. 2nd edit., rev. By T. P. HILDITCH, Campbell Brown Professor of Industrial Chemistry, University of Liverpool, Liverpool England. John Wiley and Sons, Inc., New York, 1947. viii+554 pp. Price \$9.00.

The second edition of this book is organized on the same plan and shares the good features and faults of the original edition, published in 1940. A brief introductory chapter serves to divide the subject of fat constitution into consideration of the component fatty acids and of the component glycerides. These two subjects are discussed systematically with appropriate sections on analytical methods and techniques and on the quantitative aspects of the compositions of the natural fats insofar as they are known. The book is exceptionally well documented with well over 2000 references to the published literature. In general, the composition of fats has been found to follow the biological and botanical orders quite closely. Accordingly, it has been found convenient to group the discussions around (1) the fats of aquatic flora and fauna, (2) the fats of land animals, and (3) the vegetable fats. Throughout the discussion of fatty acid composition there is traced a trend toward simplification both in the fats of plants and those of animals as the evolutionary scale is ascended. A distinction between the fats of plants and animals is noted in the case of glyceride structures, in that the distribution of the fatty acids among the glycerol molecules tends to follow the rule of "even distribution" in seed fats and "random distribution" in animal fats.

The sections devoted to individual fatty acids and to individual synthetic glycerides, as well as the sections on analytical technique, will be of considerable interest to workers in the field of fat composition. However, it is in this department that the book might be considerably improved and the author should consider reediting these two chapters in the next edition.

One obvious weakness of the book is that, while much new material has been added, relatively little obsolete material has been deleted. This results in frequent contradictions. For example, isovaleric acid is repeatedly mentioned as the only known example of an acid containing a branched chain or an odd number of carbon atoms, yet other more recently published examples are given on pp. 395 and 427.

The author is enthusiastic about ester distillation as a means for fatty acid analysis. However, he states that "for the less complex and more easily separable ester mixtures the simpler Willstätter flask apparatus is entirely adequate." This apparatus, with an efficiency of not more than two theoretical plates, is quite obsolete and its use can only lead to inaccurate results. For example, a recent analysis of rape seed oil, while reporting all components to the nearest 0.1%, failed to mention Δ^11 -docosenoic acid, which is a major component present to the extent of 10-15%. The use of boiling points as a criterion of identity in conjunction with saponification numbers would help to avoid such errors. The reporting of minor components, particularly those present to the extent of 1% or less, is of doubtful validity, except in those instances where careful use has been made of efficient modern distillation equipment.

The book is excellently printed in clear, legible type but the quality of paper and binding are not quite up to prewar standards. Typographical errors are exceedingly rare. One notable error occurring in both the first and second editions is a discrepancy between the name and structure assigned to gorlic acid. Professor Hilditch is to be commended for having made another worthwhile contribution to the literature of fat chemistry.

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Dynamic Aspects of Biochemistry. By ERNEST BALDWIN, B.A., Ph.D., University Lecturer in Biochemistry, formerly Fellow of St. John's College, Cambridge. The University Press, Cambridge; The Macmillan Company, New York. Price \$4.00.

With the exception of nuclear physics, probably no branch of science has grown so rapidly during the last 20 years as has biochemistry. Almost irresistibly the various branches of biology and medicine appear now to converge into a general biochemistry. Therewith, a strong demand arose for authentic outline and rationalization of the results of these recent advances. In recognition of such a demand, many text books have tried valiantly, but not quite successfully, to fit the new into their old scheme. It is, therefore, a great relief to meet the hoped for book where the attempt is made with genuine success to build a new frame rather than to renovate the old ones.

The book by Baldwin requires an advance knowledge of elementary biochemistry. It is a broad treatment of the biochemical domain and is, therefore, of course, not snugly fitted to the need of an average medical student. Nevertheless, it is intended to be an advanced text book aiming at intelligent resonance rather than retentive memory. It addresses itself, indeed, to a mature audience.

The book is divided into two parts: the first part entitled "Enzymes"; the second and more comprehensive part entitled "Metabolism." The first part attempts briefly to describe and classify the host of enzymatic reactions, the actors in the metabolic play, as it is treated in the second part. It certainly is necessary to make a start with such a new classification, although the one proposed here may not be the best possible one. The scheme probably still leans too heavily on the *catabolic* aspects of metabolism. In due time, the enzymatic apparatus of *anabolism* will assert its characteristics more thoroughly in a final classification.

The metabolic part is introduced by a brief but keen description of the methodological "ladder" from whole animal to tissue homogenate and extract. It seems somewhat unfortunate that, in the main section, protein metabolism is treated first. A more organic picture might be obtained starting with the discussion of carbohydrate metabolism, as being the main thoroughfare wherefrom and whereto the other branches of metabolism arise and connect. Much interesting detail, however, is contained in the somewhat more inclusive treatment of nitrogen metabolism.

The book has courage and charm, and it is thoroughly enjoyable to read. It is a fine book.

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Advances in Carbohydrate Chemistry. Vol. III. Edited by W. W. PIGMAN and M. L. WOLFROM. Academic Press, Inc., New York, 1948. xxiii + 424 pp. Price \$8.50.

The first two volumes of *Advances in Carbohydrate Chemistry* established the utility and importance of this publication for the integration of knowledge in special-

ized branches of carbohydrate chemistry. The third volume of the series maintains the high standard which characterize the first two volumes. No praise can be too high for the initiative of those who brought forth this series of publications. They have made available a wealth of material for the stimulation, guidance, and help of specialists and non-specialists alike.

The text opens with a memorial biography of Rudolph Maximilian Goepp, Jr., by M. L. Wolfrom. Dr. Goepp's untimely death is regretted by all workers in the field, and it is indeed appropriate that this expression of appreciation should appear in a publication which he helped to establish.

In the first 21 pages of the book, C. S. Hudson presents a section entitled: "Historical Aspects of Emil Fischer's Fundamental Conventions for Writing Stereo-Formulas in a Plane." Unfortunately, the nomenclature of the higher sugars is relegated to a footnote in which the two systems now used are mentioned, and the fundamental differences in assigning the α and β names are not considered. In spite of this fact, the section provides an authoritative, accurate, and reliable source of information on the historical background of the conventions now used in representing stereo-formulas in a plane and for naming the sugars.

"The Structure and Reactivity of the Hydrazone and Osazone Derivatives of the Sugars," are reviewed by E. G. V. Percival. The discussion of the anhydrides of osazones and hydrazones is particularly good, but might have been improved by less emphasis on structures of historical interest now known to be in error. The author skilfully brings out the complex structural problems and points the way for much new work. (22 pp., 44 refs.)

The widespread occurrence of inositols in nature, and especially the recent discovery of a 1,3-diguandino-2,4,5,6-tetrahydroxycyclohexane group in streptomycin, have aroused great interest in biological circles in "The Chemistry and Configuration of the Cyclitols," a subject covered by Hewitt G. Fletcher, Jr. The author presents an interesting and well-organized discussion of this difficult subject in 32 pages with 107 references.

"The Trityl Ethers of Carbohydrates" are dealt with by Burckhardt Helferich. The treatment, although largely descriptive, is comprehensive and authoritative. A considerable portion of the chapter is devoted to discussion of the use of derivatives obtained by means of tritylation for the synthesis of other materials and for the determination of structure. A useful list of carbohydrate trityl ethers is included in the article. (34 pp., 104 refs.)

The chapter entitled "Glucose and The Unfermentable Reducing Substances in Cane Molasses," by Louis Sattler, will do much to clarify a page of carbohydrate chemistry which has long been filled with error, confusion, and controversy. With impartial thoroughness, the author presents the historical background concerning the myth of two 3-ketohexoses in the unfermentable substance obtained from cane molasses or from D-glucose and D-fructose by the action of alkali. The unfermentable substances which have now been isolated include anhydro sugars and minor amounts of simple substances like methylglyoxal. (16 pp., 38 refs.)

The widespread use of halogen oxidation of sugars for analytical purposes, for the preparation of industrially important materials, and for structural studies, gives interest to the article by John W. Green entitled: "The Halogen Oxidation of Simple

Carbohydrates." The author presents a comprehensive and well-organized resumé of the work in this field. (56 pp., 180 refs.)

For several years the molecular structure of cellulose as elucidated by the Haworth school has been under attack by those who believe that long, uniform chains of anhydro-D-glucose units do not adequately account for the presence of certain relatively labile linkages. In an interesting article entitled: "The Molecular Structure of Cellulose," Jack Compton presents the experimental basis for both the conventional ideas and the new concepts. (34 pp., 161 refs.)

Under the heading: "Isotopic Tracers in the Study of Carbohydrate Metabolism," Samuel Gurin presents a timely and instructive discussion of the available isotopes and the use of isotope tracers in the field of carbohydrate chemistry. One is impressed by the striking results which have been obtained in the study of CO_2 assimilation in plants and microorganisms, and in the study of the synthesis and oxidation of carbohydrates in animal tissues. (22 pp., 76 refs.)

In a section entitled: "Products of the Enzymic Degradation of Starch and Glycogen," Karl Myrbäck presents an excellent resumé of our knowledge concerning the structure of starch and its enzymic degradation. Particular emphasis is placed on the interpretation of the facts in terms of an irregular-branched structure of amylopectin and a linear structure for amylose. The discussion of the limit dextrins obtained from various starches and enzymes is particularly thorough. (60 pp., 107 refs.)

Recognition of the unique immunological properties of the complex polysaccharides of *M. tuberculosis* has led to extensive research with respect to methods for the separation, purification, and identification of the serologically active polysaccharides. In an article entitled: "The Polysaccharides of *Mycobacterium Tuberculosis*," M. Stacey and P. W. Kent present an exceedingly interesting account of the work in this field. A table of the polysaccharides that have been separated is included in the article. Our knowledge concerning the structures is fragmentary but rapid progress is being made. (26 pp., 84 refs.)

In the last chapter of the book, R. N. Lemieux and M. L. Wolf from report on the investigations which led to the determination of the chemical structure of streptomycin. The course of scientific progress is marked by signal discoveries which bring to fruit the academic knowledge painstakingly developed over many years. Elucidation of the structure of streptomycin illustrates this fact. In 3 short years, a page of carbohydrate chemistry was written which can well be an inspiration for generations to come. Many persons past and present contributed to this outstanding accomplishment, but it is appropriate that the report in *Advances in Carbohydrate Chemistry* should be written by two of the main contributors.

The authors of this volume are to be congratulated for presenting their topics in an inspiring and well-integrated fashion. The compilation and classification of special knowledge in the several branches of carbohydrate chemistry will save future students and researchers much time. No investigator who deals with carbohydrate chemistry can afford to be without this book.

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Minimal Protein Requirement for Growth in the Rat ¹

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INTRODUCTION

In 1915, Osborne and Mendel (1) reported that young rats can complete their growth satisfactorily when a diet contains 18% of casein along with the essential nonprotein components of the diet. Thirty years later, it is generally believed that the young rat requires about 10–12% of a high quality protein in the ration, although 18% protein diets are commonly fed (2).

With the purpose of determining the minimal protein requirement, the growth and reproduction of rats was observed on diets ranging from 19.1 to 4.7% protein, supplied by polished rice, red kidney beans, and casein. The following considerations were taken into account:

1. The preparation of diets containing different concentrations of a protein mixture, in which the limiting factor was known to be protein, and not some vitamin, mineral, or other nutrient.

2. The characterization of the protein in some way, *i.e.*, by "true" digestibility and biological value (3), which would permit the prediction of the minimal requirement of other proteins.

3. The detection of the limiting essential amino acid (1), if possible.

4. The observation of the minimal protein level that supports growth in several generations of rats, such as occurs on an adequate diet under similar laboratory conditions and is believed to be normal growth (4) for the colony.

5. The measurement of the coefficient of protein utilization for

¹ Published with the approval of the Director of the School of Tropical Medicine and the Director of the Agricultural Experiment Station of the University of Puerto Rico. A cooperative project.

growth (5), for it is probable that the maximum occurs under conditions of protein underconsumption (5.6).

6. Relationship between protein and caloric intake.

Data obtained on reproduction and lactation will be reported elsewhere.

EXPERIMENTAL

Diets

The protein of the diets was supplied by a mixtrue of polished rice, 56.4 parts; red kidney beans, 28.2; and casein, 15.4. Vitamins, minerals, and cornstarch were added. The diets contained about 1.5% fat and 3.4 cal./g.

The composition of the diets which were prepared from current supplies every week, are given in Table I. The rice was ground but not cooked. The beans were

TABLE I
Composition of the Rice-Beans-Casein Diets

	Per cent protein						
	19.1	16.7	14.3	11.9	9.5	7.1	4.7
Constituents ^a							
Polished rice	48.4	42.4	36.3	30.2	24.2	18.1	12.1
Red kidney beans	24.2	21.2	18.1	15.1	12.1	9.0	6.0
Casein (technical)	12.8	11.2	9.6	8.0	6.4	4.8	3.2
Cornstarch	11.6	22.2	33.0	43.7	54.3	65.1	75.7
Salt mixture ^b	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Cod liver oil	1.0	1.0	1.0	1.0	1.0	1.0	1.0

^a Synthetic vitamins added as supplement to each kg. of diet: thiamine HCl, 1 mg.; pyridoxine HCl, 1 mg.; riboflavin, 4 mg. 1 mg. α -tocopherol was given orally every week to second and third generation males. Vitamins supplied by Hoffmann-LaRoche, courtesy of Dr. E. Sevringhaus.

^b Modified Hawk-Oser (7) salt mixture, with calcium carbonate substituted for calcium citrate.

soaked, cooked, dried, and ground, and proximate analyses of the prepared beans were then made. Percentage composition of the diets was calculated. From time to time, proximate analyses of the prepared diets were made; there was good agreement between calculated and observed values.

Procedure

The rats were descendants of Wistar albino rats brought to the School of Tropical Medicine in 1927. For several generations they had been receiving a diet containing

25.2% protein², similar to diet I(4), which was apparently adequate for growth and reproduction in the rat.

Young rats from females on the 25.2% protein diet were separated at the age of 21 days. Members of the same litter were distributed in groups of 6–12 animals on each of the protein levels. Control rats were given the 25.2% protein diet. Males and females were housed separately in small stationary cages set upon screens, 3 in a cage. Food consumption was recorded. Observations were also made of the growth of second and third generation rats on the diets.

The experiments were carried out under the conditions of a tropical marine climate.

DISCUSSION

"True" Digestibility and Biological Value

The "true" digestibility and biological value of the N of the diets were determined by a modified N metabolism method (3) described by Goyco and Asenjo (8). The N metabolism of 10 growing males was measured. The first and fifth metabolism periods of 7 days each, with a preliminary period of 3 days on the diet, were standardization periods, the first one on a low-protein egg diet, the fifth on a low-N diet. The

TABLE II
*Coefficients of "True" Digestibility and Biological Value
of the N of the Rice-Beans-Casein Diets*

	Mean of 10 determinations
(a) TN of diet, per cent	1.58
Body weight, initial, g.	102
Body weight, final, after 7 days, g.	108
(b) Daily food intake, g.	6.75
(c) Daily food N, mg.	(a × b) 106.6
(d) Daily fecal N, mg.	27.0
(e) Daily "metabolic" N/g. food, mg.	1.5
(f) Daily "metabolic" N in feces, mg.	(b × e) 10.1
(g) Daily food N in feces, mg.	(d - f) 16.9
(h) Daily food N absorbed, mg.	(c - g) 89.7
Coefficient of "true" digestibility	(h/c × 100) 84.1
(i) Daily N in urine, mg.	40.7
(j) Daily "endogenous" N/100 g. body weight, mg.	17.1
(k) Daily "endogenous" N in urine, mg.	(j × mean body wt.) 18.0
(l) Daily food N in urine, mg.	(i - k) 22.7
(m) Daily food N retained, mg.	(h - l) 67.0
Biological Value	(m/h × 100) 74.7
Net utilization of protein: "True" digestibility × biological value	62.8

² Protein diet (25.2%): polished rice, 48.5 parts; soybean, 25.0; dried brewers' yeast, 10.0; casein, 7.5; vegetable oil, 6.0; salt mixture, 2.0; cod liver oil, 1.0.

experimental rice-beans-casein diet, containing 9.9% crude protein (dry weight), was given during the second metabolism period; during the third and fourth periods, the rats received certain rice-beans diets not pertinent to this discussion.

Computations of the coefficient of "true" digestibility and of biological value are summarized in Table II. The biological value, 74.7, lay within the limits expected for biological values of protein of mixed diets (9).

Methionine Deficiency

All rats which were given 9.5%, or more, of dietary protein survived the period of rapid growth. Of 12 male rats receiving 4.7% protein, only one survived until the age of 84 days. The rest fell suddenly ill without loss of body weight, and died. They presented, grossly, the massive hepatic necrosis that is associated with methionine deficiency (10,11). On 7.1% protein, 4 of 8 males and 1 of 12 females also died suddenly, and gross liver lesions were observed.

Subsequently, the addition of 0.3% of DL-methionine to the diet containing 4.7% protein permitted better growth and enabled the rats to survive beyond the experimental period without the onset of liver damage. Second generation rats from females receiving 14.3 or 16.7% protein were used. The 4 rats receiving methionine in the diet survived the experimental period without evidence of liver lesions. All 25 male and female litter-mate controls died at a mean age of 44 days and presented severe liver necrosis. The limiting amino acid of the rice-beans-casein diet is, therefore, methionine, a finding to be expected in casein and legume diets (12).

These observations bore out Osborne and Mendel's statement (1) that, when the content of any essential amino acid in a protein is relatively low, the deficiency will manifest itself, not on high protein diets but on diets of low protein concentration, in which the amount of amino acid available will become insufficient for growth and maintenance.

Some alopecia was observed in about 20% of the females and 40% of the males. Although the percentage tended to increase with diminishing protein, the distribution appeared to be influenced by factors other than protein concentration of the diet.

Further evidence that the diets were limited by protein rather than by vitamins was obtained by supplementing them, in addition to cod liver oil, thiamine, pyridoxine, and riboflavin, with the following:

TABLE III

Body Weight of Rats at 84 days of Age on Rice-Beans-Casein Diets

	Per cent protein							
	24.8(4)	25.2	19.1	16.7	14.3	11.9	9.5	7.1
<i>Males</i>								
Number of det.	29							
Mean	269							
Probable error	±3.4							
<i>First generation</i>								
Number of det.		6	6	12	12	12	8	4
Mean		285	275	263	248	220	168	106
Probable error				±3.7	±2.6	±3.5		
<i>Second and Third generations</i>								
Number of det.		12		6	21	16		
Mean		260		255	206	190		
Probable error		±3.3			±2.4	±2.6		
<i>Females</i>								
Number of det.	47							
Mean	183							
Probable error	±1.6							
<i>First generation</i>								
Number of Det.		6	6	12	12	12	12	11
Mean		208	200	195	191	183	154	113
Probable error				±3.2	±2.5	±0.8	±3.2	±4.4
<i>Second generation</i>								
Number of det.		22		6	18	14		
Mean		185		192	163	132		
Probable error		±1.8			±1.5	±2.4		
<i>Third generation</i>								
Number of det.		13		5	15	9		
Mean		183		187	168	144		
Probable error		±2.5			±2.5			

niacin, Ca pantothenate, inositol, choline, PABA, folic acid, and vitamins E and K. When these substances were added, neither growth nor food consumption of the rats were altered.

Growth

Since standards of growth need to be defined (13), growth in this report will be considered normal if it is similar to that which has been observed in 4 generations of rats, under the given laboratory conditions, on an adequate diet containing 24.8% protein. This growth is similar to that reported by Smith and Bing (14).

Growth data are presented in Table III. The data of the rats on the 24.8% protein diet (4) were combined because there was no significant difference in growth between generations. Table III shows that, in the control rats on 25.2% protein, there was a significant difference in growth between rats of the second and third generations, and those of the first. Since the rats were reared under similar laboratory conditions on similar diets, it was thought that controllable experimental variables might be operating. There was no evidence of seasonal variation.

The control diet 25.2% protein and the rice-beans-casein diets, containing 16.7%, or more, of protein, supported normal growth in several generations of rats. Rats receiving 14.3% protein from the 21st day grew approximately as well as the controls, but the growth of second and third generations, on the diet from the time of conception, was significantly retarded. Although the mean body weight at 84 days of age of first generation females was 191 g., it was, respectively, 163 and 168 g. in the second and third generation females (Table III).

With further reduction in dietary protein, the rate of growth decreased in rats given the diets from the 21st day. The retardation was not significant in the case of females receiving 11.9% protein from the 21st day, but the mean body weight at 84 days of second and third generation females on the diet was 132 and 144 g., respectively.

It appears, then, that under the laboratory conditions given, the minimal protein concentration of the rice-beans-casein diet for growth in the rat is not greater than 16.7, nor less than 14.3%, of the diet.

Food Consumption

During the 9-week period, from the 21st to the 84th day, the mean daily food consumption of the male rats on the 16.7% protein diet was

12.5 g. and of the females, 10.7g. The amount of food eaten daily was about 7 g. during the first week; 10 g. during the second; 12 g., the third and thereafter, for females; 14 g., the fourth and thereafter, for males. Less food was eaten by the rats on diets containing 9.5%, and less, of protein, but no evidence of hunger was observed in their behavior.

Protein Efficiency

Data are presented in Table IV for a 28-day period beginning at the time when the rat weighed 60 g. This is the period arbitrarily selected by Osborne, Mendel and Ferry (5) for their determination of the coefficient

TABLE IV
*Caloric Intake and Protein Efficiency of Rice-Beans-Casein Diets
During 28-Day Period after 60 g. in Body Weight*

	Per cent protein					
	19.1	16.7	14.3	11.9	9.5	7.1
Males						
Number	6	12	12	12	8	
Mean body weight at end in g.	186	180	173	149	116	
Mean gain in g.	126	120	113	89	56	
Mean B.S. area ^a in cm ² .	281	276	270	251	225	
Mean food intake in g.	341	343	343	310	262	
Mean number of Cal. daily	41	42	42	38	32	
Cal./100 cm ² . B.S.	14.7	15.1	15.4	15.0	14.1	
Mean protein intake in g.	65	57	49	37	25	
Gain/g. protein in g.	1.9	2.1	2.3	2.4	2.3	
Females						
Number	6	12	12	12	12	11
Mean body weight at end in g.	159	156	153	142	116	93
Mean gain in g.	99	96	93	82	56	33
Mean B.S. area ^a in cm ² .	261	258	254	246	225	204
Mean food intake in g.	325	318	316	309	263	216
Mean number of Cal. daily	40	39	38	38	32	26
Cal./100 cm ² . B.S.	15.2	15.0	15.1	15.2	14.1	12.8
Mean protein intake in g.	62	53	45	37	25	15
Gain/g. protein in g.	1.6	1.8	2.1	2.2	2.3	2.1

^a Weight³ × 11.36.

cient of protein utilization for growth, g. gain in body weight/g. of protein eaten. It is the period of rapid growth and permitted, in these experiments, a preliminary period of about 7 days for adjustment of the animal to the protein concentration of the diet.

In Table IV the maximal protein efficiency does not occur at a definite protein concentration, as reported by Osborne, Mendel and Ferry (5) but through a range of concentrations, as observed by Barnes, Maack, Knights and Burr (15); in fact, it may be verified by calculations from data given elsewhere by Osborne and Mendel (1). The range was apparently wider in females and the value somewhat lower (16). The maximal protein efficiency, as determined with males on diets containing 14.3, 11.9, and 9.5 % protein, under the conditions given, was 2.3.

Block and Mitchell (17) correlated values for protein efficiency and net utilization of protein that were obtained from the literature. In this report, the maximal protein efficiency, 2.3, and the net utilization of protein, 62.8, have been determined for the same protein mixture. Therefore, assuming a direct relationship between the maximal protein efficiency and the net utilization of protein, it was possible to calculate the maximal protein efficiency of an ideal protein, namely, $2.3/0.628 = 3.7$. Similar values may be calculated from the data of Kao, Adolph and Liu (18) on sweet potato protein and of Adolph and Cheng (19) on certain mixed cereal proteins. Barnes, Maack, Knights and Burr (15) determined the maximal protein efficiency of egg protein to be 3.80, but it must be remembered that the experimental conditions were not the same.

Since it is probable that the essential amino acids are most economically used when the supply is restricted (5.6), and since the maximal protein efficiency is observed on the rice-beans-casein diets containing 9.5-14.3% protein, it would appear that these diets did not meet the growth requirement for protein.

Caloric Intake

The average caloric intake/100 cm.² of body surface area, during a 28-day period beginning at a body weight of 60 g., is shown in Table IV. For males, the maximal caloric consumption occurred on the rice-beans-casein diet containing 14.3% protein. Lower values appeared on either side of the maximum. Barnes and Bosshardt(20) found that the greater caloric consumption per unit body surface area occurred at the level

of maximal protein utilization. It is evident in Table IV that the relationship does not apply to females.

Net Protein Requirement for Growth

The minimal protein concentration which supports normal growth in several generations of rats on the rice-beans-casein diet was approximately 16.7%. This diet contained 1.5% fat. Since an increase in the fat content of a diet has the physiological effect of a reduction in the protein concentration and since, according to French, Black and Swift (21), the fat concentration of the diet does not significantly alter the nitrogen utilization, the protein content of the diet is expressed on a caloric rather than on a percentage basis. Net protein is suggested as a useful approximation, which takes into account the digestibility and biological value of the protein.

The 16.7% protein diet contained 7.8 TN/Cal. With a value of 84.1 for "true" digestibility of the protein, this diet was equivalent to one containing 14.0% digestible protein, or 6.6 mg. digestible TN/Cal. With a biological value of the protein of 74.7, the diet was equivalent to one containing 10.5% net protein, or 4.9 mg. net TN/Cal.

It will be interesting to see whether approximately 4.9 mg. net TN/Cal. of other proteins or protein mixtures is the minimum for growth. The results of Barnes, Maack, Knights and Burr (15) with egg protein are suggestive. They observed that on low-fat whole egg protein diets, the maximal protein efficiency occurred at 9.9% dietary protein. Rats on this diet grew nearly as well as those on the higher protein diets.

SUMMARY

Growth and food consumption of rats was observed on diets ranging from 19.1 to 4.4% protein, supplied by a certain mixture of polished rice, red kidney beans, and casein. Protein was apparently the limiting factor of the diets.

1. Methionine was the limiting amino acid of the protein mixture.
2. The minimal protein concentration which supports growth in several generations of rats, such as that obtained on an adequate diet, was 16.7%. This diet contained 7.8 mg. TN/Cal.
3. Since the "true" digestibility of the protein mixture was 84.1, the diet was equivalent to one containing 14.0% digestible protein, or 6.6 mg. digestible TN/Cal.

4. Since the biological value of the protein was 74.7, the diet was equivalent to one containing 10.5% net protein, or 4.9 mg. net TN/Cal.

5. Rats receiving 14.3% protein from the 21st day grew approximately as well as the controls, but in successive generations they grew less rapidly. With further reduction in the dietary protein, the rate of growth decreased in rats that were given the diets from the 21st day.

6. The maximal protein efficiency occurred in diets containing 14.3, 11.9, and 9.5% protein, and was 2.3. The maximum protein efficiency of an ideal protein, under the conditions given, would presumably be 3.7.

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Enzymatic Hydrolysis of Soybean Protein ¹

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INTRODUCTION

During the war, soybeans were considered as a possible supplementary source of protein for the occidental peoples. However, our fundamental knowledge of the quality and the biological value of these proteins was limited. Only one study of the *in vitro* digestion of glycinin, the globulin from the soybean, had been made in this country, and that in 1916 when the available methods were inadequate (1). At the request of the Committee on Proteins of the Food and Nutrition Board of the Nation Research Council, the action of proteolytic enzymes on crude soybean protein, such as might be present in supplementary foods, was investigated.

EXPERIMENTAL

Materials. The crude soybean protein² had a nitrogen content of 15.66% (moisture- and ash-free basis), 96.2% of which was precipitable by trichloroacetic acid.

The pepsin and trypsin were commercial preparations.³ These were used in the ratio of 1 part enzyme to 5 parts protein. The pepsin was dissolved in a mixture of sodium hydroxide and hydrochloric acid of the same ionic strength as that used for the solution of the protein and the trypsin in a 0.2 *M* borate buffer (pH 8.33). The peptidases

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² This protein was supplied through the courtesy of J. K. Seulke of the A. E. Staley Co., Decatur, Illinois. According to his description, "The protein had been extracted with a very mild alkali and then precipitated with acid. The concentration of acid had been carefully controlled in order to prevent as far as possible the denaturing of the protein. The protein represents 75% of the original material and is in the order of 95% pure."

³ Pepsin (1:2000) and trypsin (1:110) were obtained from the Digestive Ferment Corp., Detroit, Michigan.

were obtained by the customary glycerol extraction of the intestinal mucosa of hogs (2). The activity of these extracts was tested with DL-leucylglycine and DL-leucylglycylglycine (3).

Methods. As the starting point for the study of hydrolysis by enzymes, it was necessary to determine the free acidic and basic groups liberated by complete acid hydrolysis. The procedure is summarized in Table I.

TABLE I

Complete Hydrolysis of Crude Soybean Protein

The protein mixed with 20 times its weight of HCl (1:1) was heated in a boiling water bath 24 or 48 hr., samples A and B, respectively. After the removal of the major portion of HCl by 3 successive evaporations, and the humin by filtration, the filtrates of A and B contained 1.388 and 1.606 mg. N/ml., respectively. The acidic and basic groups have been determined by the Willstätter and Linderstrøm-Lang titrations (4,5), corrected for chlorides, ammonia, and humin. All values represent averages of 2 or more determinations.

	Acidic groups		Basic groups	
	<i>m. eq./100 mg. N</i>	<i>per cent total N</i>	<i>m. eq./100 mg. N</i>	<i>per cen. total N</i>
Hydrolyzate A	5.504	77.1	5.335	74.7
B	5.432	76.1	5.232	73.3
Protein ^a	1.016	14.2	0.511	7.2
Increase A	4.488	62.9	4.824	67.6
B	4.416	61.9	4.721	66.1
Average increase	4.452	62.4	4.772	66.8

^a Averages of 6 titrations on 3 different samples.

To secure a solution of the water-insoluble protein for peptic digestion, it was dissolved in sodium hydroxide and then rapidly acidified with hydrochloric acid. In all experiments the ionic strength of the digest, the protein, and the active pepsin controls were identical.

For subsequent digestion by other enzymes, aliquots of the peptic digest and the controls were neutralized, adjusted to the appropriate pH with sodium hydroxide and the enzymes added.

All digestions were carried out at 30°C., with toluene as a preservative. The changes in pH (glass electrode) observed during the digestion by pepsin, trypsin, and peptidases, were from 1.51 to 1.63, 8.15 to 7.60, and 8.00 to 7.90, respectively.

The extent of the enzymatic hydrolysis was followed by both the Willstätter and the Linderstrøm-Lang titrations (4,5). Here, as well as for the determination of the groups liberated upon complete acid hydrolysis, these titrations were carried out on a semimicro scale.

The course of the peptic digestion was also followed by the change in the amount of nitrogen precipitated by trichloroacetic acid. Aliquots (2 ml.) of the digests or controls were pipetted into an equal volume of 20% trichloroacetic acid and shaken frequently

during the first 3 hr. After standing 18–24 hr., the precipitate was transferred to 5 cm. filter paper (Whatman 40), washed 3 times with 10% trichloroacetic acid, and covered with a watch glass. After the precipitate had drained for 24 hr., the paper containing the precipitate was folded and pressed between pads of filter paper to remove the adherent acid solution. The nitrogen content of the precipitate was determined by the microKjedahl procedure (6). All values were corrected for the blank obtained with filter paper which had been washed with similar quantities of trichloroacetic acid solution.

RESULTS AND DISCUSSION

Complete Hydrolysis

The analyses of the acid hydrolyzates are presented in Table I. A fair agreement has been obtained for the total number of titratable groups (after correction for chlorides, humin, and ammonia) in the 2 hydrolyzates A and B, heated 24 and 48 hr., respectively, as shown by a comparison of the acid groups of A with those of B, *etc.* Expressed as per cent of total nitrogen, the difference between the acidic groups of the 2 hydrolyzates is 1.0%, and between the basic groups, 1.4%.

On the other hand, comparison of the titratable acidic groups with the basic groups of the same hydrolyzate shows a greater difference, 2.4 and 2.8% for hydrolyzates A and B, respectively (Table I, Columns 3 and 5). This discrepancy is even greater when the *increases* in acidic groups are compared with those of the basic groups, 4.7 and 4.2% for A and B, respectively and furthermore the basic groups are higher than the acidic groups. These differences cannot be accounted for by the humin. In both hydrolyzates the ratio of the nitrogen in the humin to that in the filtrate was 0.0098 or 0.069 m.eq./100 mg. of protein nitrogen.

The possibility that this difference might be due to presence of acid used in the preparation of this protein has been eliminated by dialyzing a fine suspension of the solid protein in distilled water until the test for sulfate ions was negligible. The titratable groups in the dialyzed product were identical with those for the untreated protein, calculated on moisture-ash-free basis.

The nonamino nitrogen, 34% of the total nitrogen, as indicated by the complete hydrolysis, is higher than would be expected on the basis of the arginine, lysine, tryptophan and histidine content of glycinin (18.1%) (7). The value obtained in this study may be indicative of peptides resistant to hydrolysis, of destruction of amino acids or of a marked difference in the amino acid composition of the proteins present

other than glycinin, and of the substances not precipitated by trichloroacetic acid. As the hydrolyzate gave a negative biuret test, any peptides present must have been chiefly dipeptides.

This increase in titratable groups after complete hydrolysis, equivalent to 62-67% of the total nitrogen, is lower than that reported for some proteins. Among the highest values reported are those of ovalbumin, lactalbumin and β -lactoglobulin 72.8, 72.4, and 79.5-81.1%, respectively (8,3,9). The values for lactoglobulin are for the acidic and basic groups calculated from the data of Hotchkiss (9). Our value of 62-67% is lower than that obtained for the acid hydrolysis of soybean oil meal protein, 74%, a preparation which was probably a more complex mixture than the one used in this study (10). It is similar to that for casein, 69-70% of the total nitrogen as measured by the ninhydrin reaction and 64-65% by the nitrous acid reaction (11), and to the liberated titratable basic groups of the globulin of the tung nut, 63% of the total nitrogen (12).

Enzymatic Hydrolysis

Examples of the results of digestion by pepsin as followed by changes in the amount of nitrogen precipitated by trichloroacetic acid are presented in Table II. The speed with which detectable changes occur is striking, as is shown particularly in Exp. 3. In Exp. 2, although a series of samples was removed immediately after the addition of the pepsin, the 2 for precipitation by trichloroacetic acid were the fifth and sixth of the series, but the exact time was not noted. On the other hand, in Exp. 3, the samples for precipitation were removed first and the exact time was recorded. A decrease of almost 16 and 20% in the nitrogen of the precipitate occurred in 1 and 2 min., respectively, whereas with the titrations (not recorded in the table) no significant differences were detectable in a similar period.

These data are similar to those obtained for β -lactoglobulin, but are at variance with those for other proteins. As calculated from the data of Haugaard and Roberts (13), for the digestion of β -lactoglobulin by crystalline pepsin at 30°C., 55.8, 30.5, 18.8, 15.2, and 13.0% of the total nitrogen were in the trichloroacetic acid precipitates at the end of 5, 60, 120, 180, and 243 min., respectively. In contrast to our data, soybean oil meal protein digested with commercial pepsin for 48 hr. at 40°C. contained 13% of the protein in precipitable form according to Evans

TABLE II

Changes in the Amount of Nitrogen Precipitated by Trichloroacetic Acid during the Peptic Digestion of Crude Soybean Protein

All values are averages of duplicate determinations, except where noted, and those for the digests have been corrected for the precipitate obtained from the enzyme controls.

Experiment 2			Experiment 3		
Period of digestion	Precipitable N		Period of digestion	Precipitable N	
	Protein control	Peptic digest		Protein control	Peptic digest
<i>hrs.</i>	<i>per cent total N</i>	<i>per cent total N</i>	<i>hr.</i>	<i>per cent total N</i>	<i>per cent total N</i>
0+	95.7	59.8 ^a	0.016 (1 min.)	96.8	81.0
0.5		40.0	0.033 (2 min.)		75.2 ^b
1.0		25.2	1.0		24.5
2.0		18.2	3.0		13.7
4.0		12.4	18.3		6.6
24.0		6.2	24.0		5.9
45.0	96.1	5.3	48.0		4.6
69.0		5.6	67.5	94.3	4.4
91.0		5.3	164.0	92.9	4.6
145.0	94.3	2.4			

^a These samples were taken immediately after the removal of 4 samples for other determinations and the exact intervals of time between mixing and removing the samples were not observed.

^b Single samples, but the amount of precipitate was such that 2 aliquots of the Kjeldahl digest were distilled.

(10). In a casein digest obtained on hydrolysis by crystalline pepsin at 40°C. for 36 hr., 10% of the nitrogen was still in precipitable form (11). These differences can hardly be ascribed to variations in temperature.

The course of all the enzymatic hydrolyses, as measured by the liberation of titratable groups, was typical. A summary of the data for the maximal hydrolyses is presented in Table III. In the experiments where both the groups were measured, the differences in the increases in the acidic and basic groups produced by a single enzyme preparation, calculated as per cent of total nitrogen, were practically equivalent, 0.0–1.8% and for the sequence of enzymes, 0.3–2.3% with one exception (Exp. 3, peptidases). On the other hand, a comparison of the

TABLE III

Summary of Enzymatic Hydrolysis of Crude Soybean Protein

The acidic and basic groups have been determined by the Willstätter and the Linderström-Lang titrations, respectively (4,5). The values are averages of duplicate determinations corrected for the initial titrations and enzymes. See Table I, Columns 3 and 5 for the base lines: *i.e.*, the increases obtained upon complete hydrolysis by acid.

Experiment no.	Sequence of enzymes	Period of digestion	Acidic groups		Basic groups	
			In terms of		In terms of	
			Total N	Complete hydrolysis	Total N	Complete hydrolysis
		hr.	per cent	per cent	per cent	per cent
1	Pepsin	216	14.1	22.7		
2	Pepsin (a)	169	14.1	22.7		
	Trypsin (b)	141	20.0	32.1		
	(a) + (b)	310	34.1	54.8		
3	Pepsin (a)	164	13.3	22.9	14.3	21.4
	Trypsin (b)	118	18.2	29.1	18.6	27.8
	(a) + (b)	282	32.5	52.0	32.9	49.2
	Peptidases (c)	96	16.5	26.5	12.8	19.2
	(a) + (b) + (c)	378	49.0	78.5	45.7	68.4
4	Pepsin (a)	164	14.1	22.6	13.6	20.3
	Peptidases (c)	92	29.5	47.2	27.7	41.5
	(a) + (c)	256	43.6	69.8	41.3	61.8
5	Pepsin (a)	164	14.1	22.7	13.6	20.3
	Trypsin (b)	144	17.4	27.9	17.6	26.3
	(a) + (b)	308	31.5	50.5	31.2	46.6
	Peptidases (c)	92	18.8	30.1	17.1	25.6
	(a) + (b) + (c)	400	50.3	80.6	48.3	72.2

enzymatic hydrolyses, expressed in terms of the groups liberated upon complete hydrolysis, shows a greater deviation from equivalency of these titratable groups.

The digestion by pepsin in 5 separate experiments was equivalent to 14 (13.6–14.3)% of the total nitrogen as measured by the two different titrations or 21 (20.3–22.9)% of the groups liberated by acid hydrolysis.

When commercial trypsin was added to peptic digests, the subse-

quent cleavage was more extensive than that produced by pepsin. The average increase for both the acidic and basic groups was equivalent to 18% of the total nitrogen and for the combined action of pepsin and trypsin, a total of 32%, or approximately 50% of the acid hydrolyzable linkages.

The peptidases in an extract of intestinal mucosa hydrolyzed the peptic-tryptic digests to almost the same degree as did trypsin, 17-18% of the total nitrogen, bringing the average for the combined action of the three groups of enzymes to 49% of the total nitrogen or 75% of the value obtained with complete hydrolysis. The value for the basic groups in Exp. 3 (12.8% of total nitrogen or 19.2% on the basis of complete hydrolysis) has been omitted because of the lack of alignment with the other values, an apparent experimental error. Where the peptidase preparation acted directly on the peptic digest without previous action of trypsin (Table III, Exp. 4), the total increase in titratable groups due to the action of pepsin and peptidases was smaller than that effected by the pepsin-trypsin-peptidase sequence, approximately 7 and 10% less in terms of total nitrogen and acid hydrolyzable linkages, respectively.

These results for peptic hydrolysis are somewhat lower than those reported for peptic digests of lactalbumin (3) and crystalline ovalbumin (8,14), in which the respective increases in amino nitrogen were 18.6 and 25% of the total nitrogen, or 26 and 34% of that produced by acid hydrolysis. In these two studies, as in the present one, the peptic digestion was allowed to proceed until no further hydrolysis was perceptible. On the other hand, under the conditions of Winnick's experiments with casein (11), where the peptic digestion was but 36 hr. only 8.8% of the nitrogen was liberated as amino groups. For a similar period of digestion of soybean protein, the increases were equivalent to 11% of the total nitrogen. In a study of unheated soybean oil meal protein (10), the period of peptic digestion was only 48 hr. and the liberation of amino groups was equivalent to 13% of the total nitrogen which is comparable to that for our soybean protein for the same period, 11-12%.

For the total enzymatic hydrolysis, comparisons may be made with lactalbumin, ovalbumin, and β -lactoglobulin. In the case of lactalbumin (3) and ovalbumin (8) hydrolyzed by pepsin, the enzymes in extracts of pancreas and intestinal mucosa, the total increase in amino groups was equal to 68 and 70% of the total nitrogen or 94 and 96% of the linkages

capable of being hydrolyzed by acid, respectively. With β -lactoglobulin digested by the enzymes in crude extracts of pancreas and intestinal mucosa, the cleavage was 97% of that obtained by acid hydrolysis, the highest value recorded in the literature (9). For our crude soybean protein the average for the total enzymatic hydrolysis was equivalent to 49% of the total nitrogen or 75% of the hydrolysis effected by HCl. With the unheated soybean oil meal protein, only 35% of the total nitrogen was liberated by the combined action of pepsin, trypsin and "erepsin" (10). In that study, in contrast to our experiments, the periods of digestion were only 48 hr. The difference was not in the peptic digestion, but in the digestion effected by the other enzymes. With the soybean oil meal preparation, autoclaving increased the hydrolysis effected by trypsin, but, even so, the total enzymatic digestion was but approximately half of that obtained in this study.

It may be concluded that while the enzymatic hydrolysis of crude soybean protein under the conditions of this study is not as great as that observed by others with ovalbumin, β -lactoglobulin, and lactalbumin, the cleavage is extensive and similar to that reported for casein.

SUMMARY

1. The acid and the enzymatic hydrolyses of soybean protein have been studied, with measurement of the liberation of acidic and basic groups by the titrimetric methods of Willstätter and Linderström-Lang. The course of peptic digestion was also followed by changes in the amount of nitrogen precipitated by trichloroacetic acid.

2. Peptic hydrolysis of soybean protein resulted in a rapid and striking decrease in the amount of nitrogen precipitable by trichloroacetic acid. After 1 min., the nitrogen precipitated was diminished by 16%, and after 2 min., by 20%. The average increase in titratable acidic and basic groups was equivalent to 14% of the total nitrogen, or 21% of that obtained upon complete hydrolysis by acid.

3. The average liberation of titratable groups by the action of the proteinases of commercial trypsin following peptic digestion was equivalent to 18% of the total nitrogen, or 32% for the combined effect of pepsin and trypsin, *i.e.*, 50% of the groups liberated by acid hydrolysis.

4. The average hydrolysis of the peptic-tryptic digests by the peptidases in an extract of intestinal mucosa was equivalent to 17-18% of the total nitrogen, bringing the total enzymatic digestion to 49% of the total nitrogen, or 75% of that obtained upon complete hydrolysis.

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Oxygen Production by Illuminated Chloroplasts Suspended in Solutions of Oxidants

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INTRODUCTION

The photochemical evolution of oxygen by isolated chloroplasts suspended in solutions of ferric oxalate was first reported by R. Hill (1,2). To obtain pressures measurable by the usual manometric techniques ferricyanide was also required, but in the absence of ferric oxalate, ferricyanide was reported to be ineffective as an oxidant for the reaction (3). It has been found, however, that chloroplasts evolve oxygen in the presence of ferricyanide without requiring ferric oxalate (4). The same result was found by Warburg and Lüttgens (5) who had previously reported the evolution of oxygen by illuminated chloroplasts suspended in solutions of *p*-benzoquinone. As a result of these findings an investigation of the possibilities of using other common oxidants was undertaken. To the reagents may now be added chromate and several oxidation-reduction indicators. The stoichiometry and the rates of oxygen evolution with these reagents have been investigated and the results are reported here.

METHODS

Chloroplasts were isolated from leaves of *Spinacea oleracea* obtained at a local market by macerating the leaves with a blender in 0.5 *M* sucrose (0°C.), followed by filtration through muslin, and then by centrifugation for 5 min. at *ca.* 800 *g*. The sedimented chloroplasts were suspended in a small volume of 0.5 *M* sucrose and stored at 0°C.

Gas evolution was determined using Warburg manometers with double-armed vessels. Three-tenth ml. of the chloroplast suspension (containing 0.2–0.3 mg. of

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chlorophyll) and 0.2 ml. of 10% KOH were placed in the separate sidearms. The oxidant to be tested, in 0.1 *M* pH 6.5 potassium phosphate buffer, was placed in the main space of the vessel, and the system flushed with nitrogen. The light system and the intensity were the same as used previously (4). The length of the illumination period was usually 45 min. In each case the pH of the mixture was determined after checking for gas evolution, thereby ensuring that the lack of gas production was not due to an excessively high or low pH. With each set of determinations a control using *p*-benzoquinone or Hill's solution was made to determine the activity of the chloroplasts being used.

RESULTS

Gas production was observed with the following reagents: potassium chromate, sodium metavanadate, phenol indophenol, and *o*-cresol indophenol. In the case of metavanadate (0.0013 *M*) the rate of gas evolution was too low to warrant further investigation. Oxygen production was identified by absorption using phosphorous in the side arm of a duplicate vessel in the cases of potassium chromate and phenol indophenol.

The following reagents at the respective concentrations given caused negligible or no gas evolution photochemically: 0.002 *M* molybdate, 0.002 *M* bromate, 0.0054 *M* chlorate, 0.007 *M* tetrathionate, 0.003 *M* tungstate, 0.003 *M* hypochlorite, 0.0066 *M* alloxan, 0.006 *M* benzaldehyde, 0.0045 *M* permanganate, 0.003 *M* nitrate, saturated solutions of periodate and bismuthate, 0.003 *M* iodine, 0.006 *M* arsenate, 0.003 *M* persulfate, 0.0043 *M* perborate, 0.007 *M* dehydroascorbic acid and 0.003 *M* glutathione (both oxidized with iodine), Coenzyme I (2.9 mg./ml.),³ cytochrome *c* (0.02 g./ml.), 0.001 *M* methylene blue, 0.001 *M* 2,6-dichlorophenol indophenol, 0.001 *M* indigotetrasulfonate, and 0.001 *M* 1-naphthol-2-sulfonate indophenol.

Although cytochrome *c* probably would have served as an oxidant with hydroquinone as an intermediate, no attempt was made to demonstrate this after the report by Bonner and Wildman (8) that they found no evidence of cytochrome in purified spinach leaf proteins.

When chloroplasts were tipped into the perborate solution there was an initial outburst of approximately 25 mm.³ of gas. However, on illumination, no further increase of volume was found. The mixing of chloroplasts and permanganate also resulted in an initial outburst of gas. This was probably CO₂, since, even during illumination, the outburst soon decreased to a stationary value.

The above findings confirm the result obtained by Aronoff (9) with dehydroascorbic acid. Benzaldehyde has been found by Fan *et al.* (16) to cause oxygen evolution from intact cells of *Chlorella pyrenoidosa*

³ We wish to thank Dr. Severo Ochoa of the Department of Pharmacology, New York University College of Medicine, for the sample of Coenzyme I.

under an air atmosphere, the only CO_2 available being that due to respiratory activity. If young cultures were used, extensive preillumination in the absence of substrate avoided, and iodoacetate added to prevent a concomitant dismutation of benzaldehyde, a stoichiometric relationship between the volume of oxygen evolved and the amount of benzaldehyde added was obtained. No oxygen evolution by *Chlorella* suspended in solutions of nitrate, chromate, alloxan or methylene blue was observed. Only with chromate and benzaldehyde in the group of reagents tested above, was there any difference between the results with whole cells and with isolated chloroplasts. Intact cells of *Chlorella* suspended in Hill's solution⁴ containing a limiting amount of ferricyanide evolve oxygen quantitatively in a nitrogen atmosphere if potassium hydroxide is present in the side arm to absorb all CO_2 . This is shown in Fig. 1. Similar results with *p*-benzoquinone have been re-

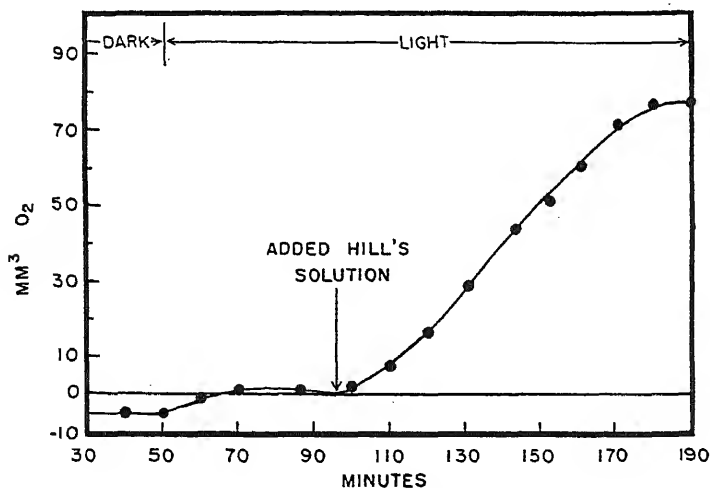


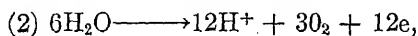
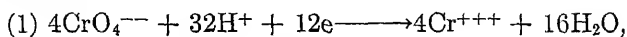
FIG. 1. Oxygen evolution by illuminated intact cells of *Chlorella* immersed in Hill's solution containing a limiting amount (15×10^{-6} moles) of ferricyanide (O_2 equivalent: 84 mm.³) pH 6.8 McIlvaine's citrate-phosphate buffer, 25°C., nitrogen atmosphere, 78 mm.³ of oxygen evolved = 93% of theoretical

ported by Warburg and Lüttgens with *Chlorella* (7) and by Aronoff with *Scenedesmus* (9).

⁴ $\text{K}_3\text{Fe}(\text{CN})_6$, 0.02 M; $\text{Fe}(\text{NH}_4)(\text{SO}_4)_2$, 0.01 M; $\text{K}_2\text{C}_2\text{O}_4$, 0.50 M; phosphate or sorbitol borate buffer, pH 6.5 0.02 M.

1. The Reaction with Chromate

Oxygen evolution was photochemical, as shown by the fact that, when the illumination was extinguished, gas evolution ceased, and was resumed when the mixture was illuminated again. Chloroplasts heated for 15 min. at 50°C. did not catalyze this reaction, showing that the enzymatic components of the chloroplasts play an active part in the reaction. On the assumption that Cr^{+6} is reduced to Cr^{+3} , the expected stoichiometry of the reaction should be the sum of the following two partial reactions:



which, combined, give:

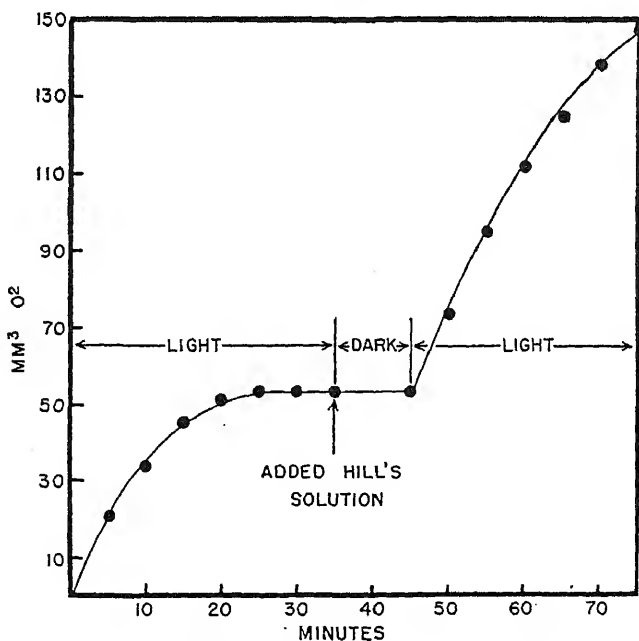
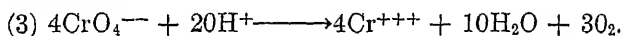


FIG. 2. Resumption of oxygen evolution by illuminated spinach chloroplasts on the addition of Hill's solution following the cessation of O_2 evolution from 6.65×10^{-6} moles of K_2CrO_4 (O_2 equivalent: 111 mm.³) pH 7.3, 0.1 M phosphate, 16°C., nitrogen atmosphere, 0.25 mg. of chlorophyll.

Thus, from 6.63×10^{-6} moles of chromate (0.00221 *M*) in the vessel one should obtain 111mm.³ of O₂. This equivalence was never realized, the yields being between 45 and 75% of the theoretical volume.

Variations in the volumes of O₂ obtained resulted with different suspensions. The failure to obtain the theoretical volume was not due to the loss of the ability of the chloroplasts to catalyze oxygen evolution as demonstrated by further evolution of oxygen when Hill's solution was added, as shown in Fig. 2. The addition of freshly isolated chloroplasts to the supernatant obtained by centrifuging out the chloroplasts from a mixture which had yielded its maximum volume of O₂, caused no further oxygen evolution. The same result was obtained when a fresh sample of chloroplasts was added from the sidearm into a mixture of chromate and chloroplasts. The effect of keeping the chloroplasts immersed in chromate solution in the dark was found to be negligible as shown by the fact that when chloroplasts were suspended in 0.00221 *M* chromate for 30 min. in the Warburg respirometers prior to illumination they gave the same rate of oxygen evolution when illuminated, as chloroplasts not immersed in a similar chromate solution but merely maintained at the same temperature. The total volumes of oxygen obtained were the same in the two cases.

The possibility that the volume of oxygen evolved bore an inverse proportionality to the concentration of some extraneous reducing material in the chloroplasts not related to the oxygen evolution reaction, was investigated by suspending different volumes of a chloroplast suspension in the same amount of chromate solution. With 6.63×10^{-6} moles of chromate (0.00221 *M*) the total volumes produced with 0.5 ml. and 0.9 ml. of suspension were 63.5 and 60.4% of the theoretical volume, respectively. This demonstrates the absence of a thermal reduction of chromate, since the final volume obtained with 0.9 ml. of suspension would have been much lower than the volume obtained with 0.5 ml. had such a reaction occurred.

The following experiment, summarized in Table I, shows the effect of chromate concentration on the total volume of oxygen evolved using equal amounts of the same chloroplast suspension. From these data it is evident that, with different amounts of chromate and equal volumes of the same chloroplast suspension, approximately the same percentage of the theoretical volume of oxygen is produced.

The possibility that a critical ratio of Cr⁺⁶ to Cr⁺³ might be responsible for the low oxygen yields, was also investigated, but proved not to be the case. The initial rate using a solution containing 0.00221 *M* chromate and 0.00221 *M* chromic sulfate was the same as that with 0.00442 *M* chromate. The total volume produced from the chromic sulfate-chromate mixture was 60.4% of the theoretical.

TABLE I

The Effect of Chromate Concentration on the Initial Rate and on the Total Volume of Oxygen Evolved by an Illuminated Chloroplast Suspension

Outline of experiment	Amount of chromate moles $\times 10^6$	Conc. of chromate M	O ₂ evolved mm. ³	Per cent of theoretical	Initial rate of reaction $Q_{O_2}^{ch}$
0.3 ml. of chloroplast suspension (0.3 mg. of chlorophyll) in 2.6 ml. total volume. 0.1 M phosphate buffer, pH 6.9, 16°C., nitrogen atmosphere.	3.98	0.00153	45.0	68	560
	5.31	0.00204	68.0	75	520
	6.63	0.00254	84.0	75	520
	7.97	0.00306	94.0	70	515
	9.30	0.00357	119.0	76	530
0.3 ml. of a different suspension from above (0.25 mg. of chlorophyll) in 3 ml. total volume. 0.1 M phosphate, pH 7.3, 16°C., nitrogen atmosphere. Dashes indicate experiment was not run to completion.	6.63	0.00221	51.0	46	852
	13.26	0.00442	101.0	45	812
	—	0.00884	—	—	852
	—	0.01768	—	—	820
	—	0.02210	—	—	856

The effect of pH on the initial rate of oxygen evolution for chloroplasts suspended in a 0.00221 M chromate solution made up with 0.10 M phosphate buffer is shown in Fig. 3 and shows the maximum rate is obtained at approximately pH 7.3.

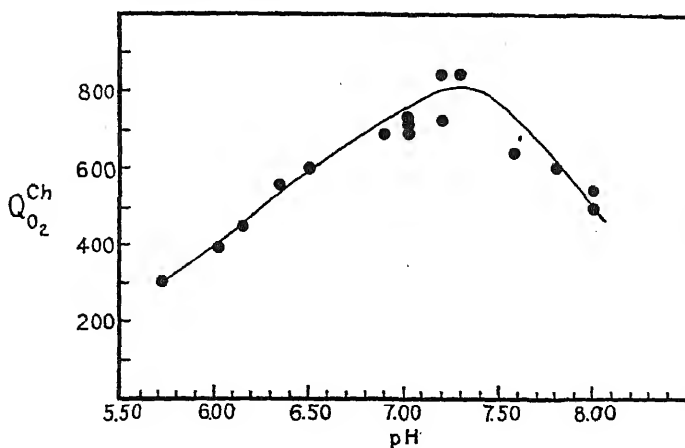


FIG. 3. The effect of pH on the initial rate of oxygen evolution by illuminated spinach chloroplasts immersed in 0.00221 M K_2CrO_4 . 0.27 mg. of chlorophyll per vessel, 16°C., nitrogen atmosphere, 0.1 M potassium phosphate buffer.

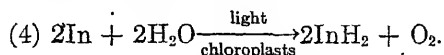
2. The Reaction with Ferricyanide

The factors affecting the stoichiometry of the reaction when complete Hill's solution is used have been dealt with in detail previously (4), the yield being 92.9% of theoretical, and, since the same number of equivalents of ferricyanide should be consumed regardless of whether the reaction mixture is ferricyanide alone or ferricyanide with ferric oxalate, an equivalence of 4Fe^{+3} to 1 O_2 was assumed.

The absence of the additional iron salts present in Hill's solution makes it possible to use phosphate buffer without the complications introduced by the precipitation of ferric phosphate. With 0.10 *M* phosphate and 0.02 *M* ferricyanide the maximum rate obtained was found to be at pH 7.1. The shape of the curve was similar to that obtained with chromate and phosphate buffer.

3. The Reaction with Oxidation-Reduction Dyes

a. Manometric Measurements. As with chromate, oxygen evolution by chloroplasts suspended in phenol indophenol discontinues when the light is extinguished, and is resumed when the chloroplasts are again illuminated. Chloroplasts heated at 50°C. for 15 min. do not catalyze such a reaction. The purity of the sample of phenol indophenol used was found to be 55% when titrated against ascorbic acid by the method of Bessey and King (10). Data showing the relation between the amount of phenol indophenol present and the volume of oxygen evolved are given in Table II, and show that the stoichiometry of the reaction is represented by the following equation:



With indicator concentrations of the range 0.00033–0.0033 *M*, the initial rate decreased 63% with this increase of concentration. This probably resulted from an increased absorption of the light by the dye, which would decrease the light intensity on the chloroplasts.

b. Reduction and Decolorization of Dilute Solutions of Various Dyes. Such indicators as 2,6-dichlorophenol indophenol and methylene blue, which are blue in the neutral region, absorb too much of the red light when used in the concentrations required for manometric measurements. This reduces the light intensity on the chloroplasts to such an extent that they will not catalyze oxygen evolution. Therefore, decolorization of dilute solutions of the indicators was investigated. Assays for purity

TABLE II

The Relation Between the Moles of Phenol Indophenol Present and the Volume of Oxygen Evolved by an Illuminated Chloroplast Suspension

0.3 ml. of chloroplast suspension containing 0.3 mg. of chlorophyll in 3 ml. total volume. 0.10 *M* pH 6.5 phosphate buffer. 16°C. Nitrogen atmosphere. * denotes a different stock solution of dye was used.

Amount of phenol indophenol present <i>moles</i> $\times 10^6$	Oxygen evolved <i>mm.</i> ³	Per cent of the theoretical volume per cent
0.99	11.0	101
1.98	22.0	101
3.46	36.0	93
3.46	40.0	104
3.46	37.0	96
3.46*	37.0	96
4.95	52.0	96
5.93*	63.0	96
		Average— 98%

were made only with phenol indophenol and 2,6-dichlorophenol indophenol. The other dyes were adjusted to concentrations such that a clearly visible color difference existed between a chloroplast suspension with the dye and a duplicate without dye. The chloroplast chlorophyll in these suspensions was 0.01 mg./ml. Distinct visibility of phenol indophenol was found in such suspensions with a concentration of 5.5×10^{-5} *M* at pH 6.5. 2,6-Dichlorophenol indophenol at pH 6.5 is blue, in contrast to red for phenol indophenol, and it was found with this reagent that one can extend the useful visual range of activity measurements down to 5.6×10^{-6} *M* dye with a chlorophyll concentration of 0.0015 mg./ml. At such a concentration this reagent has an oxygen equivalent, excluding any thermal reduction causing a decolorization, of 0.06 mm.³/ml. Such sensitivity provides an activity test for small amounts of chloroplast material. The rate of the decolorization of 2,6-dichlorophenol indophenol at pH 6.5 in phosphate buffer was approximately double that with phenol indophenol with equal volumes of the same chloroplast suspension. No decolorization of these reagents occurred if the chloroplasts had been heated previously, nor did any occur if a mixture of unheated chloroplasts and dye were not illuminated.

In Table III are given the results for the decolorization of various indicators in phosphate buffer at pH 6.6, or in borate buffer at pH 9.0

TABLE III

*The Decolorization of Dilute Solutions of Oxidation-Reduction Indicators
by Illuminated Chloroplast Suspensions*

Chlorophyll concentration: 0.01 mg./ml. 0.02 *M* phosphate, pH 6.6 or 0.02 *M* borate-KCl-NaOH buffer, pH 9.0. 20°C. Air atmosphere.

Indicator	pH	E'_0 (volts)	Decolorization
Phenol indophenol	6.6	0.254	***
	9.0	0.083	***
2,6-Dichlorophenol indophenol	6.6	0.247	***
	9.0	0.089	*
<i>o</i> -Cresol indophenol	6.6	0.217	**
	9.0	0.089	**
1-Naphthol-2-sulfonate indophenol	6.6	0.147	—
	9.0	0.003	—
Thionine	6.6	0.074	**
	9.0	-0.001	—
Methylene blue	6.6	0.024	—
	9.0	-0.050	—
Indigo tetrasulfonate	6.6	-0.027	—
	9.0	-0.114	—
Indigo disulfonate	6.6	-0.104	—
	9.0	-0.199	—

***: rapid decolorization.

**: less rapid decolorization.

*: slow or little decolorization in 10 min.

—: no decolorization in 10 min.

with an air atmosphere, and the corresponding values of E'_0 . A striking case of specificity is shown by the fact that 1-naphthol-2-sulfonate indophenol is not reduced even though its E'_0 value at pH 6.6 compares favorably in the positive range with those of other indicators which function well in alkaline solutions where the E'_0 values drop to 0.083 volts.

To the above results with dyes may be added the absence of any visible decolorization of dilute iodine or permanganate solutions by chloroplasts during a 10 min. illumination period.

DISCUSSION

The results of this investigation to determine what oxidants other than ferric oxalate, ferricyanide or *p*-benzoquinone could serve as hydrogen acceptors in the photochemical evolution of oxygen by isolated chloroplasts, have enlarged the number of suitable acceptors. It is of interest to report that suspensions of chloroplasts isolated from *Stellaria media* readily reduce phenol indophenol and 2,6-dichlorophenol indophenol without the necessity of having ferric oxalate present. That chloroplasts isolated from this species require a ferric salt, such as ferric oxalate, to catalyze oxygen evolution, was reported by Hill in his first papers announcing such a photochemical reaction.

The reduction of thionine was found to be different from that of the indophenol dyes, in that a marked but incomplete decolorization occurred very rapidly. No further decolorization was found to occur and if the chloroplast-dye mixture was removed from the light, the dye was rapidly reoxidized. Evacuation of the system in a Thunberg tube did not at any time allow the dye to be completely decolorized. In addition, only those chloroplast suspensions that gave high rates of reduction of the indophenol dyes showed any decolorization of thionine.

The problem of why chromate fails to yield the volumes of oxygen expected on the basis of a reduction Cr^{+6} to Cr^{+3} still remains to be solved. Very likely it is a property of chromate and not of the chloroplasts. There are such possibilities as: (a) the occurrence of a photochemical reduction of the chromate in the presence of an oxidizable substrate, as has been extensively investigated by Bowen *et al.* (11, 12, 13,14) and recently by Weber and Asperger (15);(b) the attainment of an equilibrium; (c) the reduction of chromate to some other state than Cr^{+3} ; (d) the evolution of oxygen from chromate rather than from water.

A significant result of this work is the fact that highly colored reagents have been found to be suitable as hydrogen acceptors. This will permit the use of extremely dilute preparations of chloroplasts, such as one might encounter during fractionation of chloroplast preparations.

ACKNOWLEDGMENTS

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SUMMARY

1. Illuminated chloroplasts suspended in solutions of chromate, or oxidation-reduction indicators such as phenol indophenol, catalyze the evolution of oxygen. This reaction does not occur if the light is extinguished or if the chloroplasts are heated at 50°C. for 15 min.

2. Oxygen evolution by chloroplasts suspended in chromate solutions is not quantitative on the basis of a reduction of Cr^{+6} to Cr^{+3} . The amounts vary between 45 and 75% of the volume expected.

3. The initial rate of oxygen evolution for chloroplasts suspended in 0.00221 *M* chromate and 0.1 *M* phosphate buffer at 16°C. is maximum at pH 7.3. The initial rate with 0.02 *M* ferricyanide under the same conditions is maximum at pH 7.1.

4. Oxidation-reduction indicators such as phenol indophenol, *o*-cresol indophenol, and 2,6-dichlorophenol indophenol, are reduced by illuminated chloroplasts in an air atmosphere. This does not occur in darkness, or in the light if the chloroplasts have been previously heated.

5. Oxygen evolution was found to be stoichiometric using phenol indophenol, two moles of the dye being reduced per mole of oxygen evolved.

6. A dilute solution of 2,6-dichlorophenol indophenol, equivalent to 0.06 mm.³ O_2 /ml. can be used to determine visually the activity of a chloroplast preparation with a chlorophyll concentration of 0.0015 mg./ml.

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Sulfur Balance Indexes of Casein in Adult Dogs with and without Addition of DL-Methionine¹

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Methionine increases the retention of nitrogen in the body of the dog and rat. Miller (11), for example, showed that DL-methionine fed to dogs maintained on a very low protein diet, has a marked protein-sparing action which becomes less striking as the reserve stores of protein are depleted. Small daily supplements of DL-methionine were found to provide a variable but large proportion of the sulfur requirement in the dogs on a very low protein diet, DL-methionine being more effective in this respect than L-cystine. Miller reported that the total urinary sulfur excretion, following the feeding of DL-methionine did not exceed the total sulfur of the amino acid supplement. A large increase in the urinary organic sulfur was observed in some of the L-cystine experiments and in all of the DL-methionine experiments. The exact nature of the organic sulfur fraction of the urine was not determined.

Allison *et al.* (3) demonstrated that the addition of DL-methionine to a protein-free diet and to diets containing casein or egg white will reduce the excretion of nitrogen in adult dogs and thus increase the retention of nitrogen in the body of the animal. They found that a definite quantity of methionine is required to produce the maximum nitrogen-sparing action, this sparing being the result of a reduction in the formation of urea nitrogen. A similar nitrogen-sparing action of methionine has been described by Stevenson *et al.* (16) in rats.

Stekol (15) showed that L- and DL-methionine sulfur were almost completely and equally well retained by adult dogs maintained on a protein-free diet, and by growing dogs maintained on low sulfur diets. Sixty-three to 71% of the sulfur from either L- or DL-methionine was retained by the adult dogs. He did not find, however, that the administration of methionine to adult dogs decreased the output of urinary nitrogen.

There is need to determine whether or not methionine will conserve sulfur as well as nitrogen in adult dogs. To do this it is necessary to

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develop some quantitative measure of sulfur retention. One of the most direct methods for the determination of nitrogen retention in the dog is the measurement of the nitrogen balance index (2). Experiments were set up, therefore, to measure sulfur as well as nitrogen balances in dogs with the hope that a sulfur balance index could also be calculated.

A concurrent study of sulfur and nitrogen balances was carried on in dogs fed casein with and without the addition of DL-methionine, the urinary sulfur being fractionated into inorganic sulfates, ethereal sulfates, and organic sulfur. In addition, the methionine and nitrogen content of the urine was determined.

It was the purpose, therefore, of this investigation to study (1) the utilization of protein sulfur and develop a concept of sulfur balance index, and (2) the effect of methionine on sulfur utilization as compared with its effect on nitrogen utilization in the dog.

METHODS

These investigations were made on normal healthy adult male dogs weighing from 7 to 12 kg. The isocaloric diets fed were prepared as described by Allison and Anderson (1). Methionine, as a powder, was added to each diet, when required, equivalent in nitrogen to 0.04 times the casein nitrogen fed. Each dog received food equivalent to 70 cal./kg. body weight/day.

The dogs were permitted to drink distilled water *ad lib*. Each dog was placed on a protein-free diet for 5 days followed by a 5-day period of a protein feeding. The protein was included in the diet by replacing an equivalent amount of calories from the carbohydrates.

The urine and feces were collected daily. The urine was acidified with HCl and collected under toluene. Carmine red capsules were used to mark the feces which were collected in friction-top cans. The last 3-day urine and 5-day fecal collections for each period were pooled and left in the refrigerator pending analysis. The fecal matter was homogenized in a Waring Blendor and diluted to 1000 ml. with distilled water. The pooled urine collections were diluted to a definite volume with distilled water. The urine and fecal samples were analyzed for total nitrogen by the Pregl micro Kjeldahl method using selenium oxychloride as the catalyst.

The fecal sulfur was determined by the Wolf and Osterberg method (17). This method was found to recover 99% of added methionine sulfur. The total urinary sulfur was determined essentially by the Benedict method (4) following neutralization of excess acidity with 10 *N* NaOH. The preliminary neutralization was necessary to recover at least 99% of methionine sulfur added to urine. The total urinary sulfate sulfur and inorganic sulfate sulfur were determined by the Polin methods (6). The difference between the total sulfur and total sulfate sulfur equals the organic sulfur. The ethereal sulfate sulfur is the difference between the total sulfate sulfur and the inorganic sulfate sulfur.

The method published by McCarthy and Sullivan (10) modified by Hess and Sullivan (7) has been adopted to determine methionine in urine. The method as presently described was found to determine at least 85% of methionine added to urine. A sample equivalent to half a day's urine slightly acidified with HCl was evaporated just to dryness in an 800 ml. beaker over a steam bath. The residue was then extracted with three 25 ml. portions of *n*-butyl alcohol. The beaker was covered during each extraction with a watch glass and heated over a steam bath for 0.5 hr. periods each. After each extraction period the solution was filtered through a pyrex glass filter, No. F, into a pyrex beaker and the combined filtrates evaporated to dryness over a steam bath. The residue was then dissolved in 25 cc. of 1 *N* H₂SO₄ and 0.5 g. of Norit A added. The Norit A was previously washed free of HCl-soluble substances. This suspension was boiled for 2-3 min. and filtered while hot through a No. 42 Whatman paper into a 50 ml. volumetric flask. The filter paper was washed 3 times with a total of 20 ml. of hot 1 *N* H₂SO₄. The solution was cooled to room temperature and the volume adjusted to 50 ml. Five ml. of this sample was used for the methionine determination. The color developed was read in a Beckman spectrophotometer at 520 m μ .

RESULTS

All the sulfur in the protein-free diet fed to the dogs was in the form of inorganic sulfate sulfur and equivalent to 0.142 g./day/m². body surface area. The surface area was calculated by the formula of Rhoads *et al.* (13). The data in Table I demonstrate that the fecal sulfur excre-

TABLE I
Average Data Obtained on Dogs 28, 31, and 70 on Protein-Free Diets and Various Dietary Levels of Protein Supplied by Labco Casein No. 8806

Dietary sulfur		Fecal sulfur	Absorbed sulfur
Inorganic sulfate sulfur	Casein sulfur		
<i>g./day/m.²</i>	<i>g./day/m.²</i>	<i>g./day/m.²</i>	<i>g./day/m.²</i>
0.142	0	0.146	-0.004
0.142	0.062	0.144	0.060
0.142	0.123	0.142	0.123
0.142	0.244	0.139	0.247
0.142	0.378	0.144	0.376

tion was equal to the inorganic sulfur content of the diet. The fecal sulfur excretion did not increase in magnitude when casein sulfur was added to the diet, a result which suggests that all the protein sulfur was absorbed by the animal.

The relationship between absorbed casein sulfur and urinary sulfur excretion in dogs 28, 31, and 70 is illustrated in Fig. 1, A, B, and C.

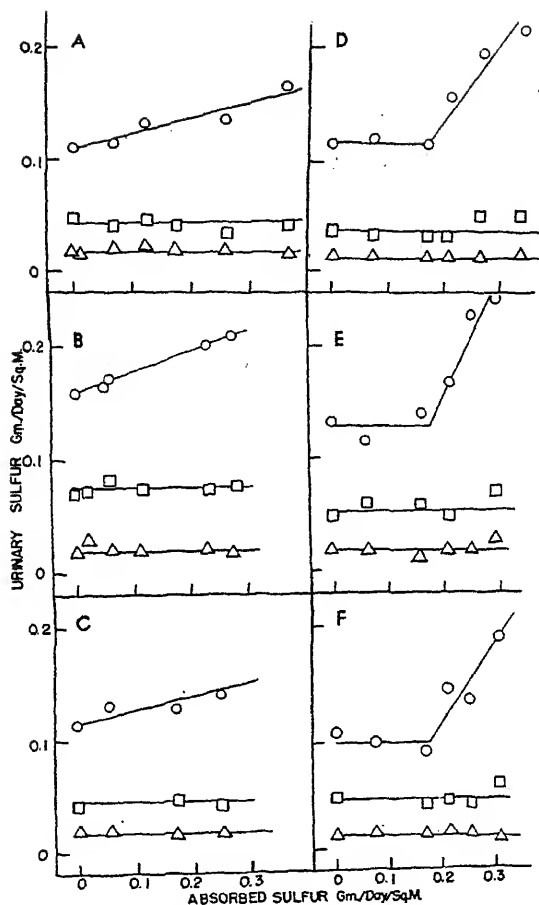


FIG. 1. Various forms of urinary sulfur plotted against absorbed sulfur in g./day/ $m.^2$ body surface area. Δ = excretion of ethereal sulfate sulfur, \square = excretion of organic sulfur, and \circ = excretion of inorganic sulfate sulfur. Data in A, B, and C from feeding dogs 28, 31, and 70, respectively, different amounts of Labco casein; D, E, and F from feeding the same dogs Labco casein + DL-methionine. (Methionine nitrogen added equivalent to 0.04 times casein nitrogen.)

These data demonstrate linear relationships between urinary sulfur and absorbed sulfur. In these experiments the excretion of ethereal sulfate sulfur and of organic sulfur is independent of sulfur intake, while the excretion of inorganic sulfate sulfur is directly proportional to absorbed sulfur. No free methionine was found in these urines.

These data can be expressed mathematically as follows,

$$US = C (AS) + US_o, \quad (1)$$

where US is the total urinary sulfur, AS is absorbed sulfur and US_o is the excretion of urinary sulfur on a protein-free diet.

Eq. (1) is in the same form as the following expression developed by Allison, Anderson and Seeley (2) showing the relationship between absorbed nitrogen (AN) and the excretion of urinary nitrogen (UN).

$$UN = (1-K) AN + UN_o, \quad (2)$$

where UN_o is the excretion of urinary nitrogen on a protein-free diet. K in Eq. (2) is the rate of change of nitrogen balance with respect to absorbed nitrogen and is called the nitrogen balance index. The C in Eq. (1), therefore, can be written $(1-K_s)$, where K_s has the same significance as K in Eq. (2). It is proposed, therefore, that K_s be called the sulfur balance index and Eq. (1) be written

$$US = (1-K_s) AS + US_o.$$

The sulfur balance indexes calculated for Labco casein from the data plotted in Fig. 1 are 0.87, 0.82, and 0.89, respectively.

The experiments on the excretion of various forms of urinary sulfur were repeated in the same 3 dogs by the feeding of casein with added methionine. The methionine nitrogen added was equivalent to 0.04 times the casein nitrogen. The excretion of the various forms of urinary sulfur in these experiments is illustrated in Fig. 1, D, E, and F. These data demonstrate that, under the conditions of these experiments, the excretion of organic sulfur and of ethereal sulfate sulfur are independent of the sulfur intake. The addition of methionine to the casein did not change the excretion of either organic sulfur or ethereal sulfate sulfur.

In the region of negative sulfur balance, methionine decreased the excretion of inorganic sulfate sulfur below the control experiments. The slope of the line $(1-K_s)$ is zero in this region, indicating that the sulfur balance index for casein plus methionine is unity. A marked increase in the excretion of inorganic sulfate sulfur occurred in these experiments when the dogs were in positive sulfur balance above 0.17 g/day/m.² The sharp inflection points of the inorganic sulfate sulfur lines occurring respectively, with dogs 28, 31, and 70 at 0.160, 0.180, and 0.170 g/day/m.² occurs when the dogs are in sulfur equilibrium. From 70 to 100% of the sulfur absorbed, beyond that required to maintain sulfur equilib-

rium, was excreted in the urine of these dogs. Thus, in the region of negative sulfur balance the excretion of sulfur is reduced by the addition of methionine to casein. The addition of methionine to casein reduced the amount of sulfur necessary to maintain equilibrium, the excess sulfur being excreted as inorganic sulfate in the urine. No free methionine was detected in the urine of these dogs, even at the highest methionine intakes.

The effect of methionine on the retention of nitrogen of the casein is illustrated by the data plotted in Fig. 2. These are average data ob-

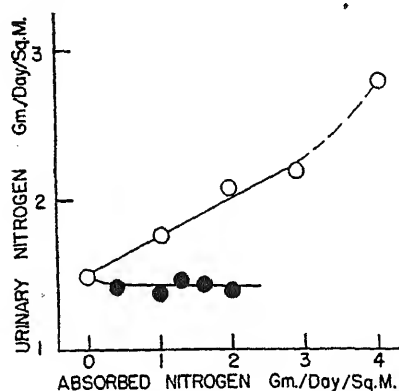


FIG. 2. Urine nitrogen vs. absorbed nitrogen in g./day/m². body surface area, average data obtained on 3 dogs. O = data from feeding Labco casein; ● = data from feeding casein plus methionine. (Methionine nitrogen added equivalent to 0.04 times casein nitrogen.)

tained on dogs 28, 31, and 70, the urinary nitrogen excretion being less in the presence than in the absence of excess methionine. The nitrogen balance index for casein is 0.74. The nitrogen balance for casein plus methionine is slightly over unity, proving that the addition of methionine to casein spares body and dietary nitrogen in the animal, a fact reported by Allison, Anderson and Seeley (3). These authors showed that methionine produces the nitrogen-sparing action through a reduction in the excretion of urea nitrogen, the ratio between ammonia nitrogen and urea nitrogen being increased. They found that a definite quantity of methionine is required to produce the maximum nitrogen-sparing action, 0.025 g. of methionine nitrogen added to 1.0 g. of casein nitrogen producing a maximum nitrogen balance index of approximately 1.5.

The data in Table II demonstrate that as the casein nitrogen intake increases the S/N ratio decreases, indicating that there is a deficiency of sulfur in the protein being fed, a fact which was originally shown by Osborne and Mendel (12), and later by Lewis (9) and Stekol (14, 15). When the deficiency is made up by adding methionine, the excess sulfur is reflected in the increasing ratio in the region of positive sulfur balance indicating saturation of the sulfur stores and increased catabolism. Johnson *et al.* (8) and Cox *et al.* (5) have shown that the sulfur stores of man are more easily filled than those of the dog, emphasizing a difference in methionine requirement of various species.

TABLE II

Average Data Obtained on Dogs 23, 31, and 70

Urinary S:N ratios found following feeding of protein-free diet and various dietary levels of protein (Labco casein) with and without addition of DL-methionine. Methionine nitrogen added was 0.04 times casein nitrogen.

Casein						
Casein nitrogen intake g./kg.	0	0.05	0.10	0.15	0.20	0.30
Urinary sulfur g/day/m. ² (S)	0.18	0.20	0.21	0.220	0.224	0.246
Urinary nitrogen g/day/m. ² (N)	1.63	2.00	2.34	2.87	2.80	3.77
S/N	0.11	0.10	0.09	0.08	0.08	0.07
Casein with addition of methionine						
Casein nitrogen intake g./kg.	0	0.02	0.05	0.065	0.08	0.10
Urinary sulfur g/day/m. ² (S)	0.16	0.16	0.17	0.21	0.24	0.29
Urinary nitrogen g/day/m. ² (N)	1.50	1.46	1.38	1.54	1.59	1.75
S/N	0.11	0.11	0.12	0.14	0.15	0.17

The relationship between absorbed sulfur and sulfur balance was found to be similar to the relationship between absorbed nitrogen and nitrogen balance, being linear in the region of negative and becoming curvilinear in the region of positive balance. The addition of methionine to casein reduced the amount of both nitrogen and sulfur necessary to maintain equilibrium in the adult dog. The production of a positive sulfur balance is the result of growth in sulfur stores. The sulfur stores were so filled in the presence of optimum quantities of methionine that an excess over the amount needed to produce equilibrium did not develop much of a positive balance but appeared largely as inorganic sulfate sulfur in the urine.

SUMMARY

1. A linear relationship was demonstrated between absorbed sulfur and urinary inorganic sulfur. The relationship between absorbed sulfur (AS) and total urinary sulfur (US) in the region of negative sulfur balance is described by the following equation:

$$US = (1 - K_s) AS + US_o,$$

where K_s is the sulfur balance index and US_o is the urinary sulfur excretion on the protein-free diet.

2. The sulfur and nitrogen balance indexes for casein averaged 0.86 and 0.74, respectively, in 3 dogs. When methionine was added to the casein (the methionine nitrogen was equivalent to 0.04 times the casein nitrogen) these indexes were increased to unity or above. Thus, the addition of methionine conserved sulfur as well as nitrogen. This conservation of sulfur was the result of a reduction in excretion of inorganic sulfate sulfur, not of ethereal or organic sulfur.

3. When the dogs were put in positive sulfur balance by feeding casein plus methionine, most of the excess sulfur not needed to maintain equilibrium was excreted as inorganic sulfate in the urine. No free methionine was excreted.

4. The urinary sulfur/nitrogen ratio decreased with increasing absorption of casein sulfur. The addition of methionine increased this ratio in the region of positive sulfur balances.

ACKNOWLEDGMENTS

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The Effects of Hyaluronidase Inhibitors on Fertilization in the Rabbit

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INTRODUCTION

The recent demonstration that various derivatives of hyaluronic acid will inhibit the viscosity-reducing effect on hyaluronic acid of hyaluronidase (1) led us to ascertain whether fertilization might be inhibited by these same derivatives. Since one of the functions of rabbit sperm appears to be the transport of the enzyme hyaluronidase which affects the dispersal of follicle cells surrounding the ova (2,3), it seems entirely likely that this dispersal would also be interfered with by the inhibitors of the enzyme's viscosity-reducing action on naturally-occurring intercellular cement.

MATERIAL AND METHODS

Semen ejaculates were obtained from tested fertile male rabbits by the use of the artificial vagina. For inseminations and experiments *in vitro*, the semen was diluted with physiological saline solution. The egg masses were washed with physiological saline solution from the fallopian tubes of freshly ovulated rabbit does. Ovulation was accomplished by the intravenous injection of pituitary gonadotrophin (4).

Egg masses were ordinarily placed in a watch glass containing an isotonic solution of the inhibitor to be tested and the saline semen suspension added. The watch glasses were placed in a moist chamber in a sterile hood at a temperature of 30-31°C., and observed from time to time. As the egg mass breaks up, the ova are liberated from the surrounding follicle cells. The time taken for complete dissolution of the egg masses and/or the separation out of the contained ova was measured. In most experiments (see Table I) an accurate sperm count was made. The concentrations of inhibitor and sperm given in Table I are the final concentrations in the watch glass preparation.

Seven different preparations, in the form of isotonic saline solutions, were used as inhibitors. Each preparation was a hyaluronic acid ester made by the methods described by Hadidian and Pirie (1). To avoid ambiguity, these are referred to by number in the text; these numbers have the following significance:

- 1 and 2: Acid-insoluble product made by nitrating hyaluronic acid of medium viscosity.
- 3 and 4: Acid-soluble product made by nitrating hyaluronic acid of low viscosity.
- 5: Derived from material similar to 3 and 4 by partial denitration with 2.5 g./l. KOH in 85% alcohol for 2 hr. at room temperature.
- 6: Hyaluronic acid of medium viscosity acetylated and then partially deacetylated by exposure for 1 hr. at room temperature to 3 g./l. KOH in 75% alcohol.
- 7: Acid-soluble product made by nitrating hyaluronic acid of medium viscosity.

Our *in vivo* studies were of two sorts; (a) those involving insemination of rabbit does with mixtures of sperm and the inhibitor in which the rabbit doe was ovulated by intravenous gonadotrophin and, (b) those in which a solution of the inhibitor was introduced intravaginally in 1 cc. of saline and natural mating (with a single ejaculation) followed. To insure ovulation, intravenous gonadotrophin was administered immediately after coitus. The does were killed 24-30 hr. later, the ova washed from the fallopian tubes, and the percentage fertilized determined. At this time fertilized rabbit ova have cleaved into 2-4 cells (5).

RESULTS

We first sought to discover whether the dispersal of follicle cells *in vitro* by sperm could be inhibited by a nitrated hyaluronic acid and if, as Hadidian and Pirie (1) have shown for hyaluronidase itself, such inhibitions were proportional to the concentration of inhibitor present. Secondly, we sought to determine whether the follicle cell dispersal and/or its inhibition were affected notably by the presence of active motile sperm. Heating the semen sample for 13 min. at 50°C. immobilized all sperm but did not destroy follicle cell-dispersing capacity. In Table I are the data of an experiment made with a low viscosity hyaluronic nitrate.

These data demonstrate: (a) that above a minimum concentration (0.1 mg./cc.) effective inhibition of follicle cell dispersal does occur when nitrated hyaluronic acid is present (Exp. 1-4); (b) that at the highest concentration, delay of dispersal is longest (Exp. 4); (c) that sperm motility scarcely affects the rate of follicle cell dispersal (Exp. 5 and 6) nor the time of action of inhibitor (Exp. 6 and 7). It should be noted that in Exp. 7 and 8, the delay in follicle cell dispersal was the same with 2 mg./cc. of inhibitor as in Exp. 4, although the sperm count was higher; this is explicable as either a chance variation or due to an upper limit of time in which, under these conditions, the most effective concentration may continue to act.

With a second nitrate, the degree of inhibition exerted when the sperm were centrifuged out of the semen and resuspended in physiolo-

TABLE I

The Inhibition of Follicle Cell Dispersal by Nitrated Hyaluronic Acid

Exp.	Inhibitor	Concentration	Treatment	Results		Remarks
				No. eggs	Mass dispersed in	
		<i>mg./cc.</i>			<i>hr. min.</i>	
1	None	—	Eggs and sperm mixed in 0.9% NaCl.	6	1 2	Total sperm in 0.6 cc. 0.20 million (Exp. 1-4)
2	4	0.1	Eggs and sperm mixed in 0.9% NaCl.	4	1 9	
3	4	0.5	Eggs and sperm mixed in 0.9% NaCl.	2	2 20	
4	4	2.0	Eggs and sperm mixed in 0.9% NaCl.	5	7 39	
5	None	—	Eggs and active sperm mixed	Cell ^a mass	1 12	Total sperm in 0.6 cc. 0.42 million (Exp. 5-8)
6	None	—	Eggs and immobilized sperm mixed	Cell ^a mass	1 5	
7	4	2.0	As in No. 5	Cell ^a mass	7 30	
8	4	2.0	As in No. 6	Cell ^a mass	7 39	

^a Ova not counted.

logical solutions was examined, comparing these data with the effects of the inhibitor on the dispersing activity of the sperm-free supernatant (Table II).

The data of Table II demonstrate: (a) that the hyaluronidase free of sperm and the hyaluronidase still present in sperm after centrifuging are both inhibited with nitrated hyaluronic acid (Exp. 9-12); (b) that the dispersal by sperm is inhibited in a buffered physiological salt solution (Pannett-Compton) as well as in an isotonic NaCl solution (*cf.* Exp. 9 and 10 with 13 and 14). Since the sperm suspensions of Exp. 13 and 14 were not the same as those of 9 and 10, a comparison of the exact times involved is not possible.

Using moderate concentration (1.1 million per watch glass) of sperm we have compared the inhibitory activity of a nitrated hyaluronic acid (No. 4) with a denitrated nitrate (No. 5) and a deacetylated hyaluronic

TABLE II

The Inhibition of Follicle Cell Dispersal by Sperm and Sperm-Free Seminal Fluid

Exp.	Inhibitor	Concentration	Treatment	Results		Remarks
				No. eggs	Mass dispersed in	
9	None	mg./cc. —	Centrifuged sperm resuspended in 0.9% NaCl	5	16.5 min.	± 6 million sperm in Exp. 9 and 10
10	1	0.9	As in No. 9	4	22 min.	
11	None	—	Supernatant of centrifuged semen in 0.9% NaCl	7	13 min.	
12	1	0.9	As in No. 11	12	51 min.	
13	None	—	Centrifuged sperm resuspended in Pannett-Compton solution	Cell ^a 3 mass	6.5 min.	Sperm not counted; not the same suspension as in Exp. 9-12
14	1	6.0	As in No. 13	Cell ^a mass	183 min.	

^aOva not counted.

TABLE III

A comparison of the Effect of Various Hyaluronidase Inhibitors on Follicle Cell Dispersion

Exp.	Inhibitor	Concentration	Treatment	Results	
				No eggs	Mass dispersed in
		mg./cc.			hr. min.
15	None	—	Eggs and sperm mixed in saline	5	0 37
16	4	0.5	Eggs and sperm mixed in saline	3	4 10
17	None	—	Eggs and sperm mixed in saline	4	0 28
18	5	0.27	Eggs and sperm mixed in saline	8	1 7
19	None	—	Eggs and sperm mixed in saline	6	0 26
20	5	0.8	Eggs and sperm mixed in saline	2	1 53
21	None	—	Eggs and sperm mixed in saline	1	0 22
22	6	0.72	Eggs and sperm mixed in saline	2	2 9
21	None	—	Eggs and sperm mixed in saline	3	0 33
22	6	2.4	Eggs and sperm mixed in saline	5	Only 20% at 5 hr. 5 min.

acetate (No. 6). The data are presented in Table III. Each odd-numbered experiment is the control for the following even-numbered one.

It is clear from the data of Table III that (a) various types of hyaluronidase inhibitors also inhibit follicle cell dispersion by sperm, and (b) that they differ somewhat in inhibitory potency. Accurate quantitative estimation of inhibiting potency is not, however, possible with these data.

TABLE IV

Follicle Cell Dispersal in Cultures with and without Phosphate in the Medium

Exp.	Inhibitor	Concentration	Treatment	Results	
				No. eggs	Mass dispersed in
23	None	mg./cc. —	2 cc. saline no sperm	Mass	$\frac{1}{2}$ left in 24 hr.
24	None	—	1 cc. saline 1 cc. PO ₄ no sperm	Mass	$\frac{2}{3}$ left in 24 hr.
25	None	—	2 cc. saline with sperm	Mass	0 43
26	4	0.50	2 cc. saline with sperm	9	3 30
27	4	0.50	1 cc. saline 1 cc. PO ₄ with sperm	4	4 7
28	4	0.67	1 cc. saline 0.5 cc. PO ₄ with sperm	6	4 8
29	4	0.50	0.5 cc. saline 1.5 cc. PO ₄ with sperm	5	3 5
30	4	0.50	Same as 29	5	4 48
31	None	—	2 cc. saline with sperm	12	0 36
32	2	1.75	Same as 31	Mass	$\frac{1}{3}$ left in 12 $\frac{1}{2}$ hr.
33	2	2.33	1 cc. saline 0.5 cc. PO ₄ with sperm	Mass	as in 32
34	2	1.75	1 cc. saline 1 cc. PO ₄ with sperm	7	4 hr. 54 min.

In Table IV we present data on the effects on inhibitor activity of varying phosphate concentrations in view of the findings (6) that phosphate may inhibit hyaluronidase activity. In each instance, whether the volume of the medium was 1.5 cc. or 2.0 cc., the total sperm count was 1.25 million. *M/15* sodium acid phosphate was mixed in the proportions indicated with saline sperm suspension.

As expected, there was very slow dispersal of the follicle cell mass in saline or saline-phosphate with no added sperm (Exp. 24 and 25) and

rapid dispersal with sperm in saline (Exp. 25 and 31). But no significant effect is demonstrated of the concentrations of phosphate used on the action of the sperm hyaluronidase. Although somewhat longer dispersal times are observed in one set of experiments in the phosphate-containing medium (compare Exp. 27 and 28 with 26), the same degree of variation in dispersal time in two experiments having identical phosphate concentrations (Exp. 29 and 30) is observed. In fact, in the

TABLE V
*Percentages of Fertilized Eggs Obtained after Insemination of Artificially
Ovulated Rabbit Females with Various Mixtures of
Sperm and Hyaluronidase Inhibitors*

Exp.	Inhibitor	Concentration	Sperm density	No. of rabbits	No. of ova	No. ova fertilized	Per cent fertilized	Remarks
		mg./cc.	millions/cc					
35	None	—	0.11	2	22	14	63.6	Significant decline in fertilization percentage in Exp. 36 and 37.
36	4	0.05	0.11	2	38	14	36.8	
37	4	0.15	0.11	2	44	4	9.9	
38	None	—	0.27	2	36	35	97.3	Significant decline in fertilization percentage in Exp. 39 and 40.
39	4	0.5	0.27	1	14	1	7.1	
40	4	1.5	0.27	2	62	2	3.2	
41 ^a	None	—	0.23	2	48	7	14.6	Decline in fertilization percentage not quite significant in Exp. 42.
42 ^a	4	2.3	0.23	2	68	1	1.5	
43	None	—	0.23	2	34	20	58.8	Significant decline in fertilization percentage in Exp. 44.
44	4	2.3	0.23	2	36	0	0.0	
45 ^a	None	—	8.00	2	20	8	40.0	Significant decline in fertilization percentage in Exp. 46.
46 ^a	4	2.0	8.00	2	61	0	0.0	
47 ^a	None	—	9.5–12.0	2	20	19	95.0	No significant decline in Exp. 48.
48 ^a	4	2.0	9.5–12.0	2	34	30	88.2	
49	1	4.0	4.25	2	38	0	0.0	
50	1	4.0	9.88	2	92	0	0.0	
51	1	4.0	22.50	2	36	14	38.8	

^a Sperm centrifuged and resuspended in saline.

experiments with preparation No. 2, the experiment with the highest phosphate concentration (34) exhibits a shorter dispersal time than those with lesser (33) or no phosphate (32). Hadidian and Pirie (1) have shown that the phosphate inhibition of hyaluronidase *in vitro* occurs in concentrations well over those normally found in body tissues and fluids, and these data would tend to corroborate their findings for the hyaluronidase action of living sperm.

Our first set of *in vivo* experiments involved mixing dilute sperm suspensions with varying amounts of inhibitor and inseminating with the mixtures. The data are summarized in Table V. Data for the control experiment for each concentration of sperm inseminated precedes in each instance the data for experiments with inhibitor. Sperm for each set of experiments was derived from the same rabbit buck.

These data demonstrate: (a) that with low sperm concentrations significant reductions in ova fertilized may be obtained with low concentrations of a potent hyaluronidase inhibitor (Exp. 35-37); (b) that, with moderate concentrations of inhibitor, nearly complete inhibition of fertilizations is had (Exp. 38-40); (c) that centrifuging sperm and resuspending them in saline tends to reduce fertilizing capacity (*cf.* Exp. 41 and 43), but that the inhibiting effect of the antihyaluronidase is nonetheless apparent with these resuspended sperm (Exp. 42 and 46); (d) that increasing the number of sperm above a certain inseminating concentration overcomes the effect of a constant concentration of the inhibitor (Exp. 49-51); and (e) that inhibitors effective against follicle cell dispersion *in vitro* are also effective *in vivo* against fertilization.

From the data of Table V it seems likely that 1 mg. of either inhibitor employed would prevent fertilization by 1-5 million sperm. On natural

TABLE VI
Effect of Various Dosages of Hyaluronidase Inhibitor on Percentage of Ova Fertilized Following a Natural Mating

Exp.	Inhibitor	Concentration	No. of rabbits	No. of ova	No. of ova fertilized	Per cent fertilized
		<i>mg./cc.</i>				
52	3	0.57	3	70	58	82.9
53	3	1.7	3	76	69	90.8
54	3	4.0	3	78	60	76.9
55	3	8.0	3	102	55	53.9
56	7	40.0	3	72	17	23.6

mating, a single ejaculation contains 50–100 million sperm. It should, therefore, follow that the introduction of low dosages of inhibitor into the vagina before a natural mating would contraceptively be ineffective but that higher doses should significantly reduce the fertilization percentage. This is illustrated in Table VI.

With inhibitor No. 3 significant reduction in ova fertilized is not apparent until a concentration of 8 mg./cc. was employed, and even with 40 mg. of No. 7 inhibition was not complete. Nonetheless, these data demonstrate that, in natural mating, as in artificial insemination, antihyaluronidases are contraceptive.

DISCUSSION

It is clear from these results that derivatives of hyaluronic acid inhibit the dispersal of follicle cells *in vitro* and are contraceptive *in vivo*. It is also clear that different derivatives vary in their potency. The investigation of the effect of these substances on the action of hyaluronidase on hyaluronic acid led to a similar conclusion, but it is not yet possible to relate the 3 methods of measuring inhibition to one another because of the different, or uncertain, ionic environments used. Thus, acetylated hyaluronic acid was found by Hadidian and Pirie (1) to be less efficient than the nitrates when tested on the hyaluronidase:hyaluronic acid reaction, but it is clear from Table III that it is as efficient when tested as an inhibitor of follicle cell dispersal. The results in this paper are only preliminary; no systematic attempt has been made to find the optimal conditions of esterification or deesterification, nor to find the optimal initial viscosity of the hyaluronic acid used in making the esters.

Although purified testis hyaluronidase or the hyaluronidase of sperm will disperse the follicular cells or cumulus cells of rabbit ova *in vitro*, it is unable to separate the corona radiata cells attached to the ova *in vitro* as observed in this laboratory and by Swyer (7). The role of hyaluronidase in fertilization has been postulated (8,9), but added hyaluronidase cannot increase the fertilizing capacity of rabbit sperm in crucial tests (10). The present study does show, however, that the probability of fertilization *in vivo* is decreased only when the concentration of hyaluronidase inhibitors overcome the number of spermatozoa present. It seems, therefore, that the probability of fertilization cannot be increased by the addition of hyaluronidase to spermatozoa unless

hyaluronidase inhibitors are present in critical concentrations in the reproductive tract.

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SUMMARY

Various hyaluronic acid derivatives which inhibit the viscosity-reducing activity of testis hyaluronidase *in vitro* also inhibit the follicle-cell-dispersing activity of sperm suspensions or of semen hyaluronidase *in vitro*. The effect is proportional to the concentration of inhibitor. It is not significantly affected by physiological concentrations of phosphate. The presence or absence of motile sperm does not affect the degree of inhibition. When the inhibitors are added to sperm suspensions artificially inseminated, the number of ova fertilized is significantly reduced. the extent of such contraception is determined by the ratio of sperm number to inhibitor concentration. When inhibitor is placed in the vagina of naturally mated females contraception also occurs with adequate inhibitor concentrations.

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The Effect of Furacin (5-Nitro-2-Furaldehyde Semicarbazone) on the Metabolism of Bacteria^{1,2}

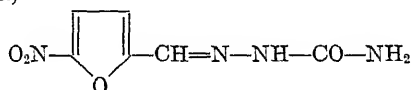
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INTRODUCTION

The antibacterial properties of furan derivatives have been recognized by several investigators. The early literature on this subject has been reviewed by Dodd and Stillman (1). Dodd and Stillman (1) investigated nitrated and non-nitrated furan derivatives. In general, it was found that the introduction of a nitro group in the 5 position of the furan ring greatly increased the antibacterial properties of the furans. Dodd, Hartmann and Ward (2) studied 5-nitrofurans with regard to toxicity for the human skin and their effect on wound healing. The compound 5-nitro-2-furaldehyde semicarbazone or furacin, having the following structural formula,



was found to be the best of the 5-nitro compounds investigated for topical application. Dodd (3), on further investigation of furacin, found that it had a wide antibacterial spectrum, being active *in vitro*, and in some cases *in vivo*, against a large variety of gram-negative and gram-positive bacteria.

Cramer and Dodd (4) studied the effect of furacin on the growth curve of *Staphylococcus aureus*. Furacin prolonged the lag phase of

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growth but not the rate of reproduction once initiated. Other nitrofurans, however, depressed the rate of growth. In a more recent study, Cramer (5) investigated the relationship of the oxidation-reduction potential to furacin action. It was found that, during the lag phase or period of inhibition, there was a poisoning of the oxidation-reduction potential. Subsequently, there was destruction of furacin accompanied by a drop in the E_h . When sufficient furacin has been destroyed or reduced, the normal enzymic activities of the bacteria are resumed, resulting in growth. Green and Mudd (6) found that organisms resistant to penicillin, streptomycin or sulfonamides showed no change in their resistance to furacin when both parent and resistant strains were tested under the same conditions.

EXPERIMENTAL

Experiments with Antagonists to Furacin

To obtain some clues as to possible sites of action of furacin, a systematic survey was made of the antagonistic activity of some important metabolites such as amino acids and vitamins. *E. coli* (No. 15) was used as the test organism, employing a simple salt glucose medium as described by Green and Sevag (7). The procedure was as follows: Sterile Klett tubes were inoculated with a washed 18 hr. culture incubated at 37°C., and the growth followed by periodic turbidity measurements in the Klett-Summerson photoelectric colorimeter over an interval of 24 hr. The following tubes were set up: 1, control medium alone; 2, medium plus antagonist; 3, medium plus furacin in final dilutions of 100,000 and 200,000; 4, medium plus furacin plus antagonist. All tubes were set up in duplicate and the experiments repeated at least twice. The reactions observed fell into the following categories: A, *Negative or no antagonism* when growths in tubes 1 and 2 were equal and the same degree of inhibition was present in tubes 3 and 4; B, *Antagonism* when growths in tubes 1 and 2 were equal and tube 3 showed significantly more inhibition than tube 4; C, *Antagonism with growth stimulation* when tube 2 showed more growth than tube 1 and tube 3 showed a significant degree of inhibition when compared with tube 4; D, *Inhibition* when tube 2 showed less growth than tube 1.

Antagonism of Amino Acids. Twenty amino acids and *p*-aminobenzoic acid were tested and classified as follows:

Antagonists

L-glutamic acid, L-lysine HCl, DL-isoleucine, DL-phenylalanine.

Antagonist stimulating growth

L-arginine.

Inhibitor

DL-norleucine.

No antagonism

DL-alanine, DL-aspartic acid, DL-citrulline, L-cysteine HCl, DL-glycine, L-histidine HCl, DL-leucine, DL-methionine, L-proline, DL-serine, DL-threonine, DL-tryptophan, DL-tyrosine, DL-valine, *p*-aminobenzoic acid.

Only 5 amino acids showed antagonism. Typical results are tabulated in Table I.

TABLE I

Antagonism of Amino Acids^a to Furacin (E. Coli in Glucose-Salt Medium)

Dilution of furacin	Control—no amino acids	DL-aspartic acid	L-glutamic acid	L-arginine	DL-isoleucine	L-lysine	DL-phenylalanine
1:200,000	16 ^b	13	99	141	106	105	55
None	105	132	105	141	114	120	106

^a 5×10^{-3} M.

^b Turbidity readings, Klett-Summerson photoelectric colorimeter (24 hr. growth).

A concentration of 0.005 M was required before the amino acids showed any antagonism. This gives a molar ratio of drug to antagonist of $\frac{2.52 \times 10^{-5}}{5 \times 10^{-3}} = 0.005$. This ratio is very low when compared to the antagonism of *p*-aminobenzoic acid for sulfonamides. However, in other systems, similarly low ratios have been observed (8). The significance of the molar ratio or Inhibition Index has been discussed by Sevag in a recent review (8) and also by Woolley (9).

Antagonism of Vitamins. Using the procedure already described, the antagonism of vitamins to furacin was determined. Thiamine, nicotinamide, calcium pantothenate, and pyridoxine showed antagonistic activity. Riboflavin and inositol gave negative results. Some typical results are given in Table II.

TABLE II

Antagonism of Vitamins to Furacin (E. coli in Salt-Glucose Medium)

Dilution of furacin	No vitamins	Thiamine 10γ/ml.	Ca pantothenate 10γ/ml.	Nicotinamide 10γ/ml.	Pyridoxine 100γ/ml.
1:200,000	24 ^a	100	99	85	76
None	105	107	102	90	98

^a Turbidity readings, Klett-Summerson photoelectric colorimeter (24 hr. growth).

Respiration Experiments

It has previously been shown that sulfonamides inhibit the respiration of bacteria (10). Sevag, Shelburne and Mudd (11) have also shown that sulfonamides inhibit anaerobic pyruvate metabolism or "carboxylase" activity. Since nicotinamide, which is related to coenzyme I and II, and thiamin which is related to cocarboxylase are

antagonistic to the action of furacin, it might be expected that furacin would inhibit the respiration of bacteria.

In the Warburg experiments, *S. aureus* was used as the test organism. Only resting cell experiments were made. Observations were made at 37°C. *E. coli* was not used except for initial experiments. However, these experiments with *E. coli* showed that furacin inhibited the aerobic dissimilation of glucose about 35%.

The bacterial suspension used in the Warburg experiments was prepared from an 18 hr. culture. The bacterial sediment was washed with *M*/30 phosphate buffer, pH 7.2, and resuspended in water. The weight of the organisms was previously determined by a calibration relating dry weight of the organisms to turbidity. Both fresh and frozen suspensions were used with similar results.

Oxygen Consumption. Oxygen consumption was determined by the "direct method" of Warburg (12). The results of a typical experiment are given in Table III. It will be observed that furacin inhibited oxygen consumption 60%.

TABLE III
Inhibition by Furacin of Oxygen Uptake (S. aureus)

Time interval	μl Oxygen consumed		Inhibition
	Control	Furacin	
<i>min.</i>			<i>per cent</i>
30	98	44	55
30	90	39	57
60	132	44	67
120	320	127	60 Totals

Contents of Warburg Cups:

Control. 1.6 mg. bacteria in 0.4 ml. water; 1 ml. *M*/15 phosphate buffer pH 7.2; 0.3 ml. 10% glucose; water 4.1 ml.; 0.3 ml. 20% KOH absorbed on filter paper in the center cup.

Furacin 1:7250. Same as control except that 4.0 ml. 1:5000 furacin was substituted for 4.0 ml. water.

Through the courtesy of Dr. D. L. Cramer of the Eaton Laboratories, a strain of *S. aureus* resistant to furacin at a dilution of 1:10,000, and the parent susceptible strain, were obtained. The resistant strain was produced by repeated subculture in increasing concentrations of furacin. The oxygen uptake of the resistant and susceptible strains was studied in the presence and absence of furacin. These results are shown in Table IV. It will be observed that there was practically no inhibition of oxygen uptake by the resistant strain. The oxygen uptake of the susceptible strain was inhibited by furacin 38%.

Using pyruvate as the substrate instead of glucose, oxygen consumption in the presence and absence of furacin was determined. These results are shown in Table V. Furacin inhibited oxygen consumption 46%.

TABLE IV
Inhibition of Oxygen Uptake by Strains Resistant and Susceptible to
Furacin (*S. aureus*)

Hourly interval	μ l Oxygen consumed				Inhibition	
	Resistant strain		Susceptible strain		Resistant strain	Susceptible strain
	Control	Furacin	Control	Furacin		
1st	96	96	103	100	per cent 0	per cent 3
2nd	91	87	94	59	4	27
3rd	52	52	69	16	0	77
4th	84	73	83	41	13	51
4 hr.	323	308	349	216	5	38 Totals

Contents of Warburg cups:

Controls. 2.2 mg. bacteria in 0.4 ml. water; 0.6 ml. 10% glucose; 1.0 ml. *M/15* phosphate buffer, pH 7.2, water 3.8 ml.; 0.3 ml. 20% KOH absorbed on filter paper in the center cup.

Furacin, 1:20,000. Same as control, except that 1.45 ml. 1:5000 furacin was substituted for 1.45 ml. water.

TABLE V
Inhibition of Oxygen Uptake by Furacin Using Pyruvate as the
Substrate (*S. aureus*)

Time interval (half hours)	μ l Oxygen consumed		Inhibition
	Control	Furacin	
1st	120	100	per cent 17
2nd	154	90	41
3rd	133	71	47
4th	131	63	51
5th	131	59	55
6th	118	42	65
3 hr.	787	425	46 Totals

Contents of Warburg cups:

Control. 5 mg. bacteria in 0.5 ml. water; 1.0 ml. *M/15* phosphate buffer, pH 7.2; 0.5 mg. MgSO_4 in 0.1 ml. water; 30 mg. neutralized distilled pyruvic acid in 0.3 ml. water; 4.0 ml. water and 0.3 ml. 20% KOH absorbed on filter paper in the center cup.

Furacin 1:7250. Same as control, except that 4.0 ml. 1:5000 furacin was substituted for 4.0 ml. water.

Anaerobic Glycolysis. The flasks were cleared of oxygen by flushing with a gas mixture containing 95% N₂ and 5% CO₂ for about 10 min. The amount of anaerobic glycolysis was determined by observing the evolution of CO₂ from NaHCO₃. No appreciable endogenous formation of CO₂ occurred in the absence of glucose. The results of a typical experiment are given in Table VI. It will be observed that furacin inhibited anaerobic glycolysis 36%.

TABLE VI
Inhibition of Anaerobic Glycolysis by Furacin (S. aureus)

Time interval (min.)	μ l CO ₂ evolved		Inhibition per cent
	Control	Furacin	
30	135	118	13
30	105	54	49
60	193	104	46
2 hr.	433	276	36 Totals

Contents of Warburg cups:

Control. 1.6 mg. bacteria in 0.4 ml. water; 0.6 ml. M/15 phosphate buffer, pH 7.2; 0.3 ml. 10% glucose, 0.2 ml. 1 M NaHCO₃, water 4.3 ml.

Furacin 1:7250. Same as control, except that 4.0 ml. 1:5000 furacin substituted for 4.0 ml. water.

TABLE VII
Inhibition of Anaerobic CO₂ Evolution from Pyruvate (S. aureus)

Time interval (half hours)	μ l CO ₂ evolved		Inhibition per cent
	Control	Furacin	
1st	200	90	55
2nd	153	61	60
3rd	233	71	70
4th	214	58	73
2 hr.	800	280	65 Totals

Contents of Warburg cups:

Control. 4.2 mg. bacteria in 0.4 ml. water; 0.7 ml. M/15 phosphate buffer pH 7.2; 0.5 mg. MgSO₄ in 0.1 ml. water; 30 mg. neutralized, freshly distilled pyruvic acid in 0.3 ml. water; 4.3 ml. water.

Furacin 1:7250. Same as control, except that 4.0 ml. 1:5000 furacin substituted for 4.0 ml. water.

"Carboxylase" Activity. These experiments were conducted under anaerobic conditions as previously described. The results of a typical experiment are given in Table VII. It will be observed that furacin inhibited "carboxylase" activity 65%.

Inhibition of Dehydrogenase Activity. The inhibition of dehydrogenases was studied by observing the retardation of methylene blue reduction by furacin with glucose as the substrate and *E. coli* as the test organism. These results are given in Table VIII.

TABLE VIII
Inhibition of the Glucose Dehydrogenase System of E. coli by Furacin^a

Mg. of <i>E. coli</i> per tube	Dilutions of furacin				Control	
	1:6,250	1:8,333	1:12,500	1:25,000	None	No glucose
0.2	31*	29	24	20	16	∞
0.1	80	77	64	45	26	∞
0.05	∞	∞	∞	120	84	∞

^a Numbers in table represent time in minutes required for 100% decolorization of methylene blue. ∞ > 24 hr.

The technique employed was that of Schnabel (13) with modifications by Mr. J. S. Gots of this laboratory. Tubes 10 × 100 mm. were used. The system contained the following: methylene blue (final concentration $5 \times 10^{-5} M$), glucose 0.001 *M*, *E. coli* (18 hr. growth washed suspension) from 0.05 mg. to 0.2 mg. per tube, furacin 1:5000 in *M*/30 phosphate buffer, pH 7.6 (1:6250 to 1:25,000 final dilutions). In all cases the total volume was brought up to 1.0 ml. with *M*/30 phosphate buffer, pH 7.6. In the controls, *M*/30 phosphate buffer, pH 7.6, was substituted for furacin. Controls containing organisms without substrate were also made. No appreciable reduction of methylene blue was observed during the period of observation with these controls. Air was excluded by layering melted petrolatum on the surface and allowing it to solidify. The tubes were placed in a 37°C. bath, and the time required for 100% decolorization of the methylene blue was recorded. It will be observed in Table VIII that furacin inhibits methylene blue reduction about 50% or more, depending on the amount of organisms and the dilution of furacin. From these results, it can be concluded that furacin, in effect, inhibits the glucose dehydrogenase system of *E. coli*. The author wishes to thank Mr. J. S. Gots for carrying out the above experiments.

Inhibition of Reaction in Which Pyridoxal Phosphate is the Coenzyme

In a previous section it has been observed that pyridoxine antagonizes furacin activity. This might indicate that some function of pyridoxine was inhibited by furacin. Recently, pyridoxal phosphate has been designated as the coenzyme of several reactions in bacteria. The conversion of pyridoxine to pyridoxal phosphate has been observed (14). Some of the reactions in which pyridoxal phosphate or another pyridoxine derivative may function as a coenzyme are: 1, the formation of indole by the

breakdown of tryptophan by means of the enzyme tryptophanase (15); 2, the decarboxylation of amino acids (16); 3, transamination (17); 4, the formation of tryptophan by the condensation of indole and serine (18,19); 5, the formation of phenylalanine in the presence of CO₂ and pyridoxine (20).

Decarboxylase Activity. Gale and his coworkers have made extensive studies of bacterial decarboxylation of amino acids (16). Using Gale's method (16), the decarboxylase activity of several strains of *E. coli* was studied. No definite evidence could be obtained to show that furacin inhibited the lysine, arginine or glutamic decarboxylases. When tyrosine was used as the substrate and *S. faecalis* as the source of enzyme, no inhibition of this decarboxylase by furacin could be demonstrated.

Gale (16) has shown that carbonyl reagents such as hydrazine, hydroxylamine, or semicarbazide, are active inhibitors of the tyrosine, lysine, and arginine decarboxylases, while the glutamic decarboxylase is not inhibited. The semicarbazide portion of furacin might conceivably inhibit the first 3 decarboxylases but no satisfactory evidence for this effect has been obtained.

Tryptophanase Activity. Indole was determined according to the method of Hapgood and Hoyle (21) with several modifications. Using *E. coli* (No. 15) as the test organism, the control during a 3 hr. period produced 10 γ of indole/10 ml. of medium (buffer and tryptophan). Under the same conditions, with a dilution of furacin of 1:5,000, 2 γ of indole was produced: furacin 1:25,000, 3 γ of indole; furacin 1:50,000, 3.5 γ of indole. The percentages of inhibition were, therefore, respectively 80%, 70%, and 65%. Further details regarding these experiments will be available in a subsequent publication.

DISCUSSION

The extended studies of many workers on the action of chemotherapeutic agents on the enzyme systems of bacteria have clearly indicated that a definition of the "mode of action" of a drug cannot be simply made in terms of the observed inhibitory reactions affected by the drug (10). The question as to whether or not the observed inhibitions represent primary reactions or are secondary to reactions of an unknown nature must always be considered. The variety of enzymatic systems inhibited by furacin is consistent with the relative nonspecificity of its action against a large variety of gram positive or gram negative bacteria.

Two recent papers, which appeared after our experiments were completed, show some similarities between the action of other furan derivatives and furacin. Clark and Dittmer (22) reported that the inhibitory action of β -2-furylalanine on *E. coli* could be antagonized by phenylalanine. The amino acids L-tyrosine and DL-tryptophan were also effective, while DL-methionine, DL-isoleucine, L-leucine, L-cysteine, L-proline, and L-histidine were slightly active. We have found that the amino acid antagonists for furacin are phenylalanine, lysine, glutamic

acid, isoleucine, and arginine. Baer (23) has observed that protoanemonin, an antibiotic containing a furan ring, inhibited the action of pyruvic oxidase. In our experiments furacin strongly inhibited the aerobic and anaerobic dissimilation of pyruvate.

As semicarbazide is an active inhibitor of enzymes containing pyridoxal phosphate, its role in the action of furacin must be carefully considered. The inhibition of tryptophanase by furacin suggests that this drug may act like a carbonyl reagent. The absence of inhibition of the decarboxylases would argue against this concept. In addition, it was found that strains of *E. coli* and *S. aureus* resistant and susceptible to furacin showed no change in their resistance to semicarbazide. This latter point would suggest that free semicarbazide plays no role in the antibacterial action of furacin. In considering the mechanism of action of furacin, it should also be remembered that there are many furan compounds which do contain semicarbazide and that have antibacterial properties (1). However, further experiments in progress on the other pyridoxal phosphate enzymes should provide a more conclusive answer to this problem.

In the experiments reported in this paper we have attempted to give a general view of the effect of furacin on bacterial metabolism. In future experiments we hope to investigate in greater detail the metabolic implications of the amino acid and vitamin antagonists to furacin. The specific enzymes involved in the inhibition of respiration, particularly the enzymes in pyruvate metabolism, will also be investigated.

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SUMMARY

Arginine, lysine, phenylalanine, glutamic acid, and isoleucine antagonize the action of furacin on the growth of *E. coli*. Pantothenate, thiamine, nicotinamide, and pyridoxine are also antagonists.

Furacin inhibits both the aerobic and anaerobic dissimilation of glucose. The aerobic and anaerobic metabolism of pyruvate is also inhibited. The glucose dehydrogenase system of *E. coli* is also inhibited by furacin.

Furacin inhibits tryptophanase activity. The lysine, arginine, tyrosine and glutamic decarboxylases are not inhibited.

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Assimilation of Tracer Carbon in the Alga *Scenedesmus*¹

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INTRODUCTION

No organic substance other than chlorophyll has as yet been unequivocally shown to take part in photosynthesis. This is true despite a recently more intensified search utilizing isotopic carbon as tracer.

The apparently simple and obvious task of using the carbon isotopes for the elucidation of the course of photosynthesis has revealed itself as an ever more complicated problem. Because photosynthesizing cells store carbohydrates much faster than they use them, it is easy to produce labeled sucrose or starch in quantity.

Hardly anyone doubts, however, that these are secondary polymerization products derived from highly reactive substances which can be used by the plant in many other ways. This first became apparent through the experiments of Ruben, Kamen and Hassid (1), who found radioactive carbon in several chemically quite different constituents of the cell after only a few mins. of irradiation. It is now recognized that all living cells seem to have the ability to incorporate carbon dioxide into organic molecules in the absence of light by way of respiratory or fermentative reactions. In most cases the carbon thus fixed appears in the form of a carboxyl group, and does not readily reach the reduction level of carbohydrate. Yet it is obvious that any intermediates or products of respiration or fermentation containing fixed tracer carbon and capable of interchange or reaction with similar components appearing in the course of photosynthesis may cause unpredictable errors in the interpretation of experimental findings. That a metabolizing cell will distribute widely any carbon dioxide fixed in the dark, the extent and direction of distribution being dependent upon the nature and rate of metabolic activities in the cell, appears to us to have been confirmed by the recent work of Benson and Calvin (2). They observed a dark fixation in numerous chemically different fractions of the cell after only 5 mins. of treatment with radioactive carbon dioxide. A conspicuous change in the distribution of tracer carbon was

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found, if the treatment with radiocative carbon dioxide was preceded by long periods of irradiation in the absence of carbon dioxide. Although their proposal that this long irradiation in the absence of carbon dioxide forms substances which can reduce carbon dioxide by dark reactions is not an impossible explanation, the assimilation can be explained on the basis that the irradiation prevented the accumulation of free carbon dioxide from respiration, and thus denuded all possible acceptors present in the cell. Tracer carbon dioxide, admitted under these conditions, presumably would be rapidly and widely distributed among the cellular constituents by metabolic processes entirely separate from the photochemical mechanism.

To prove that a compound is truly a component of the photosynthetic mechanism and to place it correctly in this mechanism, the distribution of isotopic carbon between this compound and the other cellular constituents must be determined as a function of the time of photosynthesis. Merely to isolate and identify a labeled compound after a certain period of photosynthesis does not constitute proof either of its involvement in the photosynthetic mechanism or of its photochemical origin.

The most promising attack yet made on the problem was that of Ruben *et al.* (1). They demonstrated that the major portion of carbon dioxide assimilated by barley or *Chlorella* during relatively short periods of photosynthesis was incorporated into a water-soluble substance which was easily extracted after the cells were killed. Using the carrier method, they were able to show that this substance was not one of a large number of the more familiar metabolic substrates, including most of the labeled compounds isolated by Benson and Calvin.

In confirming the observations of Ruben *et al.*, we find that a water-soluble substance appears immediately upon the start of illumination, and that it contains practically all of the labeled carbon taken up during the first moments. Its metabolic behavior differs conspicuously from that of the water-soluble dark fixation products obtained under conditions identical except for the absence of light. Studies of the distribution of tracer between this substance and the other cellular constituents as a function of duration of photosynthesis prove that it is a component in the photosynthetic mechanism. This substance has thus been chosen as the most important goal for chemical isolation and identification. For reasons of kinetics, which are the topic of this paper (the chemical evidence will be presented elsewhere), we believe it to be an "intermediate" rather than the "first product" of photosynthesis. We define an "intermediate" as any substance able to undergo photochemical reduction, whether it be a product of dark fixation or a partly reduced product of photochemical action. We define the "first product" of photosynthesis as the first substance in the photochemical mechanism which, produced wholly or partly in the light from carbon dioxide, is not susceptible to further photochemical reduction.

MATERIALS AND METHODS

The Experimental Organism

The green alga employed in these studies was the D₃ strain of *Scenedesmus obliquus*. Pure cultures were grown in inorganic medium⁴ in the presence of light, either in volumes of one liter or less or on a 100-liter scale by a method to be described elsewhere. These large volume cultures were necessary to produce material for chemical isolation, and were utilized chiefly for such purposes. However, since growth conditions were found to alter the metabolic activity of the algae, small aliquots of the 100-liter cultures were used for most of the kinetic studies recorded here.

Because the metabolic properties of unicellular algae depend strongly upon culture conditions and age, a comparison will be necessary of the algae used in the several laboratories at present studying photosynthesis. The importance of using young, vigorously growing cultures must be emphasized. The metabolic rates and quotients recorded in Table I were characteristic of our algae when the material was harvested during the rapid growth phase, not later than 6 days after inoculation.

Measuring Gas Exchange

In experiments in which the rate of assimilation of labeled carbon dioxide was followed, the Warburg manometric method was used. When larger volumes of suspension were required, aeration, irradiation, and other treatments were carried out in

TABLE I

Metabolic Rates of Scenedesmus obliquus, strain D₃

210 μ l. of cells grown in 2 days in 100-l. tank.

Weight after centrifuging, 226 mg.; after drying, 50.4 mg.

Metabolic rates measured at 25.3°C. in air with 4% CO₂.

Suspension media		0.05 M Phosphate pH 5.7	0.005 M Bicarbonate (mixed Na and K salts)
		μ l. gas/ μ l. cells/hr.	
Respiration (after illumination)	O ₂	-2.64	-2.78
	CO ₂	+2.50	+2.92
Photosynthesis at light saturation (uncorrected for respiration)	O ₂	+40.2	+34.1
	CO ₂	-38.4	-32.4
Respiration (average during 12 hrs.)	O ₂	-1.86	-1.43
	CO ₂	+1.68	+1.50
Photosynthesis at light saturation (uncorrected; after 15 hrs. in same media)	O ₂	+26.6	+36.8
	CO ₂	-24.8	-34.1

⁴ Medium: MgSO₄·7H₂O, 50 g.; NH₄NO₃, 20 g.; Na₂HPO₄, 10g.; KHCO₃, 10g.; Ca(NO₃)₂, 2 g.; FeNH₄(SO₄)₂·12H₂O, 2.4 g.; FeSO₄·7H₂O, 1.4 g.; NaCl, 1 g.; trace elements (Cu, Cd, Ni, Co, Zn, Mo, W, I, Br, B, Mn); water, 100 liters.

Erlenmeyer flasks or in suitable centrifuge tubes. Both phosphate and bicarbonate buffers at varying hydrogen ion concentrations were chosen to fit the purposes of the particular experiments.

Use of Tracer

The C^{14} was obtained from the Isotopes Division of the U. S. Atomic Energy Commission. $C^{14}O_2$ was delivered to the algae in the form of a sodium carbonate solution of known concentration, both as to total carbonate and C^{14} content. For each set of experiments this tracer stock solution was diluted by inactive carbonate or carbon dioxide to the extent desired. When used in Warburg vessels, it was tipped from a side arm into the buffered algal suspension. $C^{13}O_2$, used in a few preliminary experiments, was administered exactly as was the carbon 14.

Measuring Radioactivity

The activity of extracts and fractions was determined as such, without combustion and conversion to barium carbonate, except in a few special cases. Samples were counted with a thin mica end-window, Geiger-Müller counter of conventional design using a scaling circuit and recorder of commercial manufacture. Measurements were corrected for counter background and for self-absorption of the radiation according to the usual procedures. All measurements were relative, in that no corrections were made for the constant fraction of the radiation absorbed by the mica or lost for geometric reasons. Samples were always counted in a holder which insured reproducible orientation with respect to the counter window.

The low energy of the radiation emitted by C^{14} may introduce serious errors due to the uncertainty in the value to be used for correction for self-absorption. Even though the correction to be used for uniform samples may be computed accurately from theory, (3) only absolutely homogeneous material of uniform thickness is subject to this theoretical correction without error. When only a few mg. of sample are available, it can be shown that variations in the size and distribution of the particles of sample under the counter window can create a wide divergence between the calculated and true value necessary to correct for self-absorption. We were able to improve the accuracy in counting small samples, and to greatly facilitate the handling of minimal amounts by spreading and drying solutions in contact with disks of lens paper in a manner to be described in detail elsewhere. Nevertheless, errors in correction are more serious than the statistical errors of counting which mere patience can reduce to insignificance. The errors recorded in our data are of the statistical kind only. It should be recognized that the measurements were usually no more accurate than $\pm 5\%$, and sometimes were no better than $\pm 10\%$, because of uncertainty in regard to correction for self-absorption. In a few instances estimated errors were even greater than this. Such cases are mentioned specifically in evaluating results.

As representative of the type of data we obtained, the following experimental details will suffice:

TABLE II

Representative Data on the Distribution of Tracer Carbon between Two Chemical Fractions of Scenedesmus Cells Assimilating Radioactive Carbon Dioxide during Photosynthesis

Conditions of Assimilation

Volume of algae, 35 μ l.
 Medium, 4 ml. 0.1 M KHCO_3 (in equilibrium with air).
 Volume of Warburg vessel, 15.3 ml.
 Tracer, 0.3 ml. $\text{Na}_2\text{C}^*\text{O}_2$ solution in side arm of vessel.
 C^*O_2 available in undiluted tracer solution, 570 μ l./ml.
 Radioactivity of undiluted tracer, 95.6 counts min^{-1} (c/m)/ μ l. C^*O_2 .
 Duration of assimilation (after tipping), 150 mins.
 Uptake of CO_2 observed manometrically, 1975 μ l.

Fractionation of algae

Cells killed in hot 0.5 M acetic acid and separated into 2 fractions; acid-soluble and acid-insoluble.

Radioactivity Measurements

Acid-Soluble: (Fraction B') 40 mg. NaCl added to solution (to provide bulk) and lyophilized; residue counted as dry powder.

Weight counted, 61 mg.

Area of sample, 1 cm^2

Observed radioactivity, 24.5 ± 2.0 c/m above background.

Aliquot correction factor, 3.04.

Self absorption correction factor, 5.00.

Corrected radioactivity, $(24.5 \pm 2.0) \times 3.04 \times 5.00 = 372 \pm 30$ c/m.

Acid-Insoluble: (Fraction C')

Weight of sample, 9.82.

Weight counted, 6.93 mg.

Area of sample, 1 cm^2

Observed radioactivity, 1076 ± 17 c/m above background.

Aliquot correction factor, 1.415.

Self absorption correction factor, 2.122.

Corrected radioactivity, $(1076 \pm 17)(1.415 \times 2.122) = 3228 \pm 51$ c/m.

Tracer distribution between fractions

Acid-Soluble: $372/3600 \times 100 = 10.3\%$ of C^* .

Acid-Insoluble: $3228/3600 \times 100 = 89.7\%$ of C^* .

Equivalence between Manometric and Radioactivity Measurements

Total radioactivity in cells, 3600 c/m.

Conversion factor for undiluted tracer, $1/95.6 = 0.01047$ μ l. C^*O_2 per c/m.

Non-radioactive CO_2 available, 8960 μ l.

Radioactive C^*O_2 available, 171 μ l.

Dilution correction factor, $9131/171 = 53.4$.

Conversion factor for diluted tracer, $0.01047 \times 53.4 = 0.559$ μ l. C^*O_2 per c/m.

Volume of C^*O_2 assimilated (calculated from radioactivity found), $3600 \times 0.559 = 2012$ μ l. C^*O_2 .

Uptake of CO_2 observed manometrically, 1975 μ l.

Error, $2012-1975/1975 = +2\%$.

Fractionation Procedures

Upon completion of the desired exposure to radioactive carbon dioxide, the cell suspension was syringed from the experimental vessel into an appropriate solvent heated on a steam bath. Killing was thereby achieved in 10–20 secs. The solvent employed for killing depended upon the type of fractionation procedure. Following the technique of Ruben *et al.*, we sought first to extract the photosynthetic products with hot dilute mineral acid. In our material, a variable amount of the dry weight of the algae could be extracted along with the desired labeled substance, the amount extracted depending upon the strength of the acid and the time of heating. More satisfactory separation for kinetic studies was obtained by extracting first with hot 80% ethanol, and later separating the extracted material into benzene-soluble and water-soluble components. For chemical isolation and identification work, extraction with hot dilute acetic acid or hot water was found more suitable.

Using alcohol as a solvent, the cellular constituents were divided into 3 fractions designated as A, B and C. The alternate procedure using acetic acid gave two fractions designated as B' and C'. Because of the variation of the amount of material obtained in fraction B' with conditions of extraction, whenever acetic acid extraction was employed, exactly the same conditions prevailed in the treatment of each sample and its control during all operations. The per cent of the total assimilated tracer which was found in B after any given time of exposure was equal to that found in B' after the same time under the same conditions. Thus, these fractions were considered exactly equivalent as to labeled material although obviously differing in gross composition. Characteristics of the fractions so obtained are shown in Table III.

TABLE III

Fractionation Procedures

Alcohol Extraction

Fraction A: Alcohol- and benzene-soluble, dark green, chiefly pigments and fatty materials, approximately 21% of the dry weight of the algae.

Fraction B: Alcohol- and water-soluble, pale yellow, approximately 1% of the dry weight of the algae.

Fraction C: Insoluble residue, white powder, chiefly proteins and polysaccharides, approximately 78% of the dry weight of the algae.

Acetic Acid Extraction

Fraction B': Soluble in hot dilute acetic acid, pale yellow, from 5 to 20% of the dry weight of the algae, depending on the concentration of acid, temperature, and length of treatment.

Fraction C': Insoluble in hot dilute acetic acid, 80–95% of the dry weight of the algae.

Preliminary Investigation with $C^{13}O_2$

The amount of C^{13} which could be incorporated into the algae, even in comparatively long experiments, was not enough to insure accurate

analysis.⁵ Errors in the data ranged from 10% to more than 40%. It was recognized at an early stage that the problem of tracing the assimilated carbon through intermediates in short duration photosynthesis experiments is one that cannot be solved without a more sensitive tracer than is available in any reasonable concentration of C¹³. Fortunately C¹⁴ can be detected in a much greater dilution than can C¹³, and is thus admirably suited to the problem.

Dark Fixation of Carbon Dioxide

For theoretical, as well as for experimental reasons, it appears that carbon dioxide must be fixed by a dark reaction prior to its photochemical reduction in the plant cell. Since all living cells are now known to be capable of fixing carbon dioxide by ordinary metabolic processes, serious mistakes could be made in interpreting the results of assimilation in the light, if the nature and extent of the contribution of any dark fixation were not known. Thus, an investigation of the kinetics of the fixation of carbon dioxide in the dark, and a comparison of the substances so tagged with the properties of the substances formed in the light, are of primary importance. When Ruben *et al.* first reported a dark fixation of carbon dioxide in *Chlorella* up to a value of 0.6 volume/unit volume of cells in the course of an hour, it was believed that this was indeed the much discussed "dark pick-up" [*cf.* Rabinowitch, Chap. 8 (4)]. Recently Allen, Gest and Kamen (5) have compared relative "pick-up" and aerobic dark fixation in two genera, *Scenedesmus* and *Chlorella*. They conclude that *Scenedesmus*, the alga used in the present investigation, presents a less serious control problem in regard to aerobic dark fixation than does *Chlorella*. Our results showing the extent of aerobic dark fixation in *Scenedesmus* are presented in Fig. 1. Even after several hours, the dark exchange, which involves no net uptake of carbon dioxide, is only equivalent to the amount of carbon dioxide taken up in 30 secs. at the maximal rate of photosynthesis. Therefore, computation of the volume of labeled carbon dioxide absorbed by a sample of photosynthesizing algae on the basis of the radioactivity later found in the cells agrees within experimental error with the volume computed from manometric data (*cf.* Fig. 2). Thus, with the methods of extraction

⁵ We are grateful to Dr. S. Weinhouse, Houdry Process Corporation, for mass spectrometer analyses of the samples, and for a sample of barium carbonate enriched in C¹³.

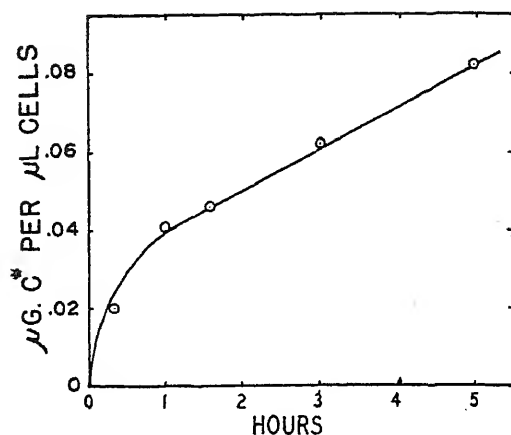


FIG. 1. Dark fixation of carbon dioxide. 105 μ L. Cells/5 ml. phosphate buffer in each Warburg vessel. Suspensions exhausted of carbon dioxide by brief illumination. Tracer added as carbonate solution from side arm. No significant volume uptake of carbon dioxide observed in any vessel during dark period.

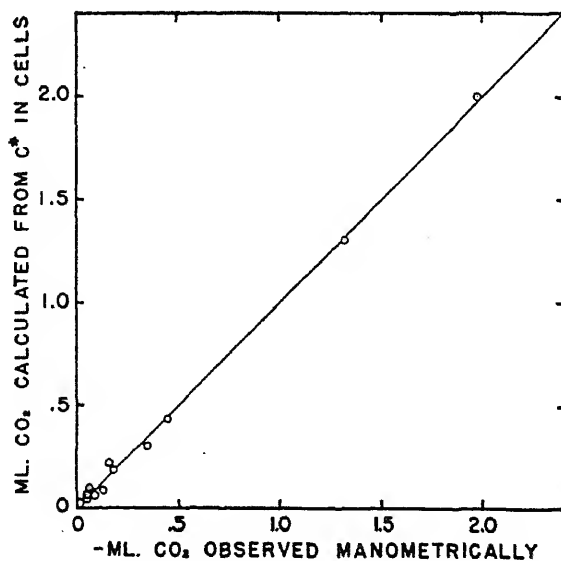


FIG. 2. Correspondence between photosynthetic uptake of carbon dioxide observed manometrically and calculated from measurements of radioactivity acquired by the algae. The method of calculation is indicated in Table II.

we employ, there is no need for an overall dark correction in photosynthesis experiments unless the assimilatory rate is reduced to less than a few per cent of its maximal value. Metabolic dark fixation might be abolished by studying the algae under anaerobic conditions. In one series of experiments we found anaerobic dark fixation to proceed about one-half as fast as aerobic dark fixation; *e.g.*, dark fixation in 1 hr.: anaerobically, 0.01 volume CO_2 /unit volume of cells; aerobically, 0.02 volume CO_2 /unit volume of cells. When cells from these dark experiments were fractionated by the alcohol procedure (*cf.* Table III), a significant difference between aerobic and anaerobic dark fixation was revealed. As Fig. 3 shows, anaerobic fixation accumulates tracer almost exclusively in fraction B, while aerobic fixation results in the tagging of all fractions. It is suggested by the time study of the fixation shown in Fig. 4 that the fixation occurs first in constituents of fraction B, and that, under aerobic conditions, the tracer is subsequently distributed to the other fractions. Because only 0.00025 volume of carbon dioxide was fixed per unit volume of cells during short time experiments, the errors in determination of the tracer content in these experiments were large. Samples having as little radioactivity as 4 c/m over background were obtained upon fractionation. However, the trend of the data is clear in that, of the total tracer fixed, the per cent found in fraction B increases with decreasing time of exposure.

The most conclusive evidence for primary fixation in fraction B is given by the experiment shown in Fig. 5. Five aliquots of a cell suspension were allowed to fix labeled carbon dioxide in Warburg vessels in the dark in an atmosphere of nitrogen for 2 hrs. The distribution of the tracer in the fractions A, B, and C at this point was determined on one aliquot (first column). Immediately after the first sample was killed, the other aliquots were washed by a stream of nitrogen until free of unfixed tracer carbon dioxide (6 mins.). That the distribution was not materially changed by this washing is shown in the second column. The 3 remaining aliquots were allowed to metabolize for 2 hrs. in atmospheres of nitrogen with 4% carbon dioxide (third column), air with 4% carbon dioxide (fourth column), and air freed of carbon dioxide by alkali in a side arm of the Warburg vessel (last column). In nitrogen, exchange of tracer carbon with gaseous inactive carbon dioxide caused a loss of tracer from fraction B, but no appreciable increase in the tracer content of the other fractions. In air (last two columns), fraction C showed a three- to four-fold increase in tracer carbon. From these data

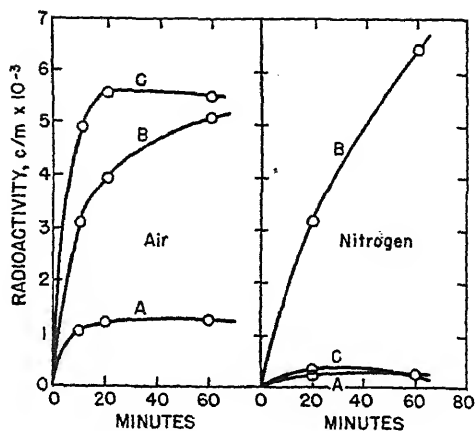


FIG. 3. Time course of dark fixation of tracer carbon dioxide in air and in nitrogen. Letters refer to fractions into which the algal constituents were partitioned. For characteristics of fractions see Table III.

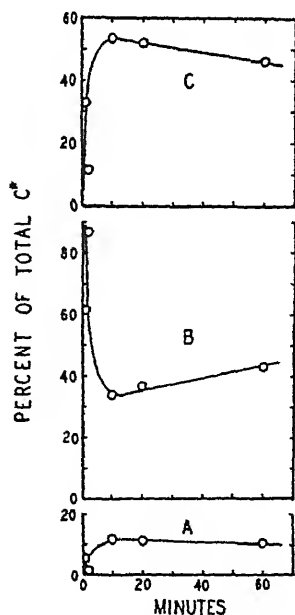


FIG. 4. Time course of distribution of tracer carbon dioxide fixed aerobically in the dark. Each point represents the tracer fixed in a particular chemical fraction of the algae as a percentage of the total tracer incorporated by all fractions.

it appears that carbon dioxide is fixed in the B fraction of the anaerobic dark fixation product in the form of a readily exchangeable group, perhaps a carboxyl; that this material is transformed into components of fraction C by respiratory action; and that, once in fraction C, it is lost by respiration rather than by exchange.

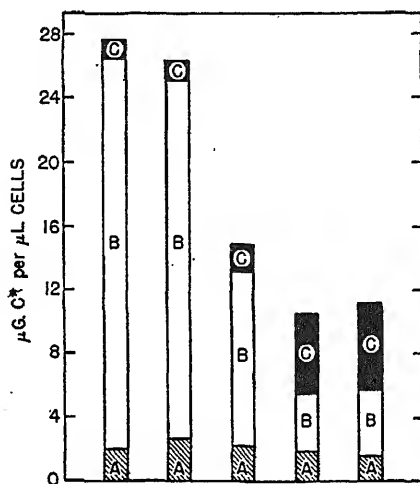


FIG. 5. Transformations of products of dark fixation of carbon dioxide. For description of experiment see text.

Another difference between the products of aerobic and anaerobic dark fixation was revealed by the insoluble silver salts obtained from the B fractions. Over 90% of the aerobically fixed tracer isolated in fraction B was precipitated by silver ion. Only 10 to 20% of the tracer fixed anaerobically in this fraction was precipitated by silver ion under the same conditions. The precipitate weights were approximately equal.

We did not attempt to identify the specific compounds into which the tracer became incorporated. The marked influence of respiration on the per cent of radioactivity precipitated by silver ion suggests that respiratory reactions may seriously complicate attempts to study the dark pick-up of carbon dioxide believed to precede the photochemical reaction in photosynthesis. On the other hand, merely to eliminate the effect of respiration by removing all oxygen or by poisoning with cyanide (5) does not allow us to distinguish with certainty between photo-

synthetic dark "pick-up" and other dark fixation since all types of fixation apparently can start, if not proceed, in the absence of oxygen.

Assimilation of Carbon Dioxide in the Light

Distribution of Tracer as a Function of Time of Illumination. In the following experiments two procedures were used preliminary to introducing the tracer carbon; the cells and the medium were either exhausted of every measurable supply of inactive carbon dioxide by an appropriate period of illumination or the cells were suspended in a carbonate buffer of known concentration. In the first case the carbon dioxide present in the radioactive carbonate solution which was added constituted the entire supply available for photosynthesis. In the

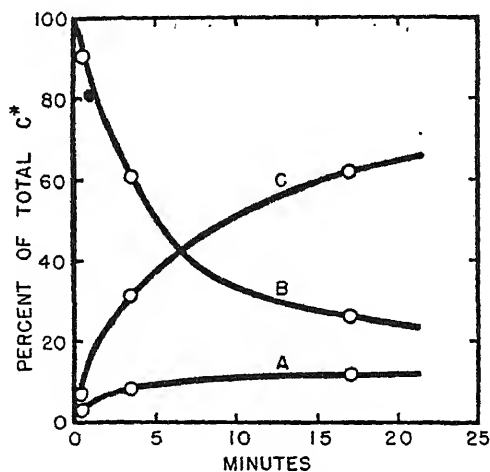


FIG. 6. Time course of tracer distribution during photosynthesis. Letters refer to the fractions into which algal components were separated. Each point represents tracer found in a particular fraction as a percentage of total tracer incorporated by the cells.

second case a dilution factor had to be taken into account in calculating the amount of isotope taken up from the buffer (as indicated in Table II). The first procedure allowed for the complete uptake of all the labeled carbon dioxide in a short time, making it possible to follow with an additional assimilation of inactive carbon dioxide. The second procedure allowed for a prolonged period of photosynthesis, with a practi-

cally constant concentration of isotope available to the algae. Fig. 6 represents the result of a series of measurements using the first method, in which the period of photosynthesis at nearly saturating light intensities was varied and the distribution of tracer was then determined as a function of this time. The data are typical of many such time studies. As in our studies of dark fixation, the water-soluble fraction B contains the highest activity/mg., and, of the total tracer fixed, the per cent found in fraction B increases with decreasing time of photosynthesis. We believe this to be proof that, in the first minutes of illumination,

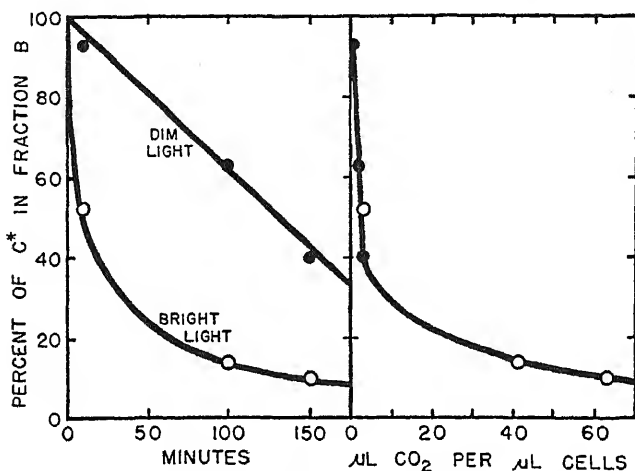


FIG. 7. Relation of tracer distribution to time of assimilation and to total photosynthesis. Data for two light intensities are plotted. Graph on left shows that tracer was shifted out of fraction B most rapidly at high light intensity. When abscissa scale of graph on left is multiplied by the respective photosynthetic rates, the graph on the right is obtained. Thus, the per cent of assimilated tracer remaining in fraction B is a function of total assimilation.

tracer carbon enters chiefly into fraction B. The material in B is later transformed into components of fractions A and C. This labeled material, which, in distinction from the dark fixation product, we call $B_{(light)}$, is apparently the first component of the photochemical mechanism stable enough to accumulate in experiments of a few seconds' duration. However, that not time of exposure to light as such, but rather the total amount of photosynthesis is the factor controlling the distribution of newly assimilated carbon is shown in Fig. 7. Since $B_{(light)}$ contains

practically all tracer assimilated during the shortest illumination periods, we may assume that the substance responsible for the activity is the same unidentified one reported by Ruben *et al.* (1). In the literature this is often referred to as the first product of photosynthesis. As stated earlier we regard it as an intermediate rather than as the first product of photosynthesis, because it is apparently susceptible to further photochemical transformation.

Properties of $B_{(\text{light})}$. Besides a chemical investigation of the properties of $B_{(\text{light})}$, an investigation of its behavior in the metabolizing cell is necessary. It is important to determine whether its transformation into the substances which accumulate in fractions A and C is accomplished by non-photochemical reactions, such as polymerization, respiration, *etc.*, or by light in a subsequent photochemical reaction.

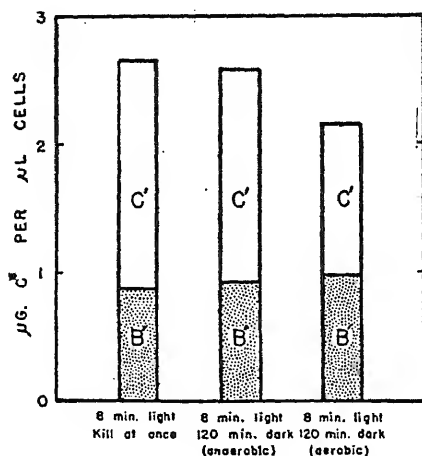


FIG. 8. Effect of aerobic and anaerobic conditions on tracer previously incorporated by photosynthesis. See Table III for characteristics of the algal fractions indicated by the letters.

Fig. 8 shows what happens if, after a short period of photosynthesis, the cells are kept in the dark and allowed to respire the newly synthesized compounds. Contrary to one's first expectations, there is no noteworthy shift of the tracer from fraction B' into fraction C', nor is there a great loss of tracer through fermentation or respiration. Respiration does remove some of the freshly incorporated tracer in the form of carbon dioxide, but the preferentially respired material seems to stem

from fraction C' rather than from fraction B' (_{light}). In several similar experiments it was found that B' (_{light}) would not exchange its tagged carbon with non-radioactive carbon dioxide in the dark. The failure to exchange or be respired suggested that B' (_{light}) is subject only to photochemical transformation. This is, indeed, the case. Under the influence of light, tracer carbon moves out of fraction B whether free carbon dioxide is present or not. However, the initial rate of this movement is 4 times as great in the presence of carbon dioxide as it is in its absence (Fig. 9).

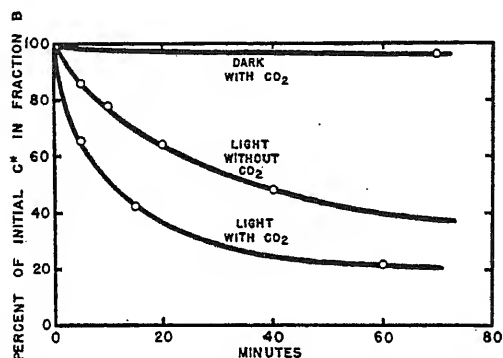


FIG. 9. Effect of light and carbon dioxide on depletion of tracer previously incorporated into fraction B by photosynthesis.

In these experiments several samples of algae were first depleted by light of any free, inactive carbon dioxide and then allowed to assimilate tracer carbon dioxide in the light for periods of 5–9 mins. After turning off the light, the small amount of tracer carbon dioxide not assimilated was removed by flushing with nitrogen for 5 mins. The vessels were then filled with nitrogen or nitrogen plus 4% carbon dioxide, irradiated for different lengths of time, the algae killed and the distribution of tracer determined. In the absence of carbon dioxide, only an insignificant amount of oxygen was developed after 40 mins. irradiation (1% of that to be expected in the presence of carbon dioxide). In the presence of non-radioactive carbon dioxide, photosynthesis proceeded at almost the maximal rate (near light saturation). In both cases an overall loss of 15–20% of the tracer originally fixed by the algae was noted in the experiments of longest duration (40–60 mins.).

That, under the influence of continued photosynthesis with non-radioactive carbon dioxide, the tracer carbon is transferred from fraction B into the other fractions is not surprising. New and interesting is the fact of its being moved by irradiation in the absence of additional carbon dioxide (compare Table IV). The properties of $B_{(\text{light})}$, exempli-

TABLE IV
Transformations of $B_{(\text{light})}$ by Continued Illumination in the Presence and in the Absence of CO_2

1. CO_2 Absent: Cells were tagged with C^*O_2 in the light for 5 mins. Light off while vessels flushed free of tracer with N_2 . Light on for times indicated. Tabular values are c/m radioactivity.

Fraction	Time of illumination without CO_2 , min.				Dark control 40 mins.
	5	10	20	40	
A	897	943	961	1027	677
B	2475	2235	1845	1368	2870
C	3430	3410	3480	3610	2550
Total	6802	6588	6286	6005	6097

2. CO_2 Present: Cells tagged with C^*O_2 in the light for 9 mins. Light off while vessels flushed free of tracer and filled with 4% CO_2 in N_2 . Light on for times indicated. Tabular values are c/m radioactivity.

Fraction	Time of illumination with C^{14}O_2 , min.				Dark control 70 mins.
	0	5	15	60	
A	847	1415	1447	1560	873
B	1710	1110	723	372	1643
C	5010	4990	4590	4300	2975
Total	7567	7515	6760	6232	5491

fied by its stability against metabolic transformation in the dark and by its ready transformation in the light, make it probable that this is not the fully reduced first product of photosynthesis, but rather an intermediate.

Steady State Saturation of $B_{(\text{light})}$. $B_{(\text{light})}$ is formed by light in the presence of carbon dioxide, and the substance so formed is transformed by light into components of fractions C and A. If the algae are subjected to continuous illumination in the presence of a constant concen-

tration of tracer, the total amount of tracer found in fraction B_(light) increases with total assimilation (cf. Fig. 10), reaching an essentially steady state after the assimilation of about 40 μ l. of carbon dioxide/ μ l. of cells. At saturating light intensities, this state is attained after about an hour of photosynthesis. The long period necessary to reach saturation of tracer in fraction B_(light) may have the trivial explanation that the fraction consists of a mixture of low molecular weight substances

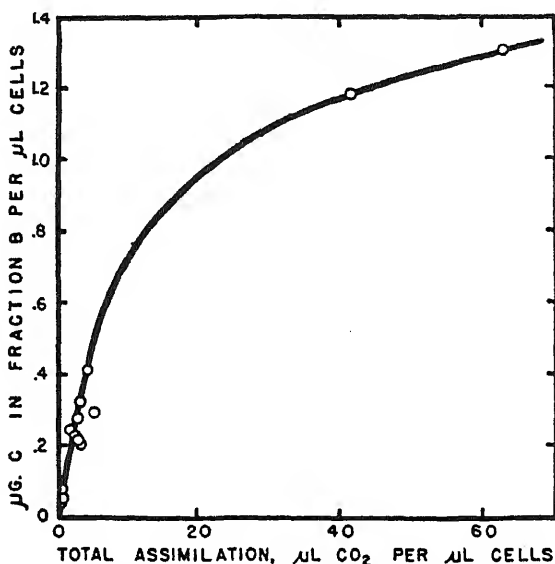


Fig. 10. Relation between tracer contained in fraction B and total photosynthetic assimilation.

which are preferentially formed from the fully reduced "first product" and thus have no direct connection with photosynthesis. An alternative explanation is that fraction B_(light) is essentially homogeneous with respect to tracer, and that the acceptor of carbon dioxide is itself a product of photosynthesis (cf. Discussion). The continued gradual rise in the tracer content of fraction B after this time can be reasonably explained by postulating the formation, either from minor side reactions in fraction B or from components of fractions A and C, of compounds possessing the solubility characteristics of fraction B but not subject to photochemical transformation. If fractions A and C are the source of

these compounds, the curve would presumably not become horizontal until the entire cell was in equilibrium with the medium; a matter of many hours.

Calculations based on the half-saturation value for tracer in fraction B (7–10 μ l. of CO_2 assimilated/ μ l. of cells) reveal that, whereas at this point only one out of every 20–25 carbon atoms in the cell has been derived from the assimilated tracer, in fraction B one out of every 3–4 carbon atoms has been so derived. This may be taken as further evidence that fraction B is the pathway through which carbon dioxide enters the cell during photosynthesis. This calculation also reveals the surprising fact that at saturation at least one-half of the carbon atoms in fraction B are derived from the added tracer.

DISCUSSION

We shall first discuss the experimental evidence presented above concerning a possible relationship between the products of dark fixation of carbon dioxide and the first substance which can be shown to have been produced photochemically by illuminated *Scenedesmus* cells. Both appear in either fraction B or B'. Thus, we have designated this group of substances as $B_{(\text{dark})}$ and $B_{(\text{light})}$. There the similarity ends. $B_{(\text{dark})}$ is formed anaerobically at an initial rate 100 times smaller than the rate of carbon dioxide assimilation at high light intensity, and its total concentration after several hours is only 1/40–1/200 of the saturation concentration of $B_{(\text{light})}$ at saturating light intensities. Under nitrogen, $B_{(\text{dark})}$ exchanges its labeled carbon dioxide with inactive carbon dioxide in the gas phase; in air, it is respired while being transformed into products appearing in the other fractions of the cell. In contrast to this, $B_{(\text{light})}$ does not readily exchange its tracer with carbon dioxide, is not respired, and undergoes transformation into components of fractions A and C only under the influence of light. While Benson and Calvin (2) and Allen, Gest and Kamen (5) were able to identify among the products of dark fixation such well known participants in respiratory reactions as succinic and fumaric acids, $B_{(\text{light})}$ has chemical properties which differ from those characteristic of the common substrates or intermediates involved in normal dark metabolism. Its remarkable stability in the unilluminated cell confirms the chemical evidence. Our results concerning the substance formed during short periods of photosynthesis are thus in agreement with the observations reported by

Ruben *et al.* (1) who were unable to identify the product of brief periods of illumination.⁶

The use of cyanide as a poison for respiration may provide conditions comparable to those found during anaerobic treatment. Therefore, our experiments on the anaerobic dark fixation of carbon dioxide have a bearing upon the interpretation of the results recently published by Allen, Gest and Kamen (5). Using cyanide they attempted to separate the respiratory dark fixation from a dark "pick-up" of carbon dioxide alleged to be associated with photosynthesis. Our results indicate that the anaerobic dark fixation is perhaps a first step in the aerobic dark fixation. The properties of the products of both aerobic and anaerobic dark fixation are at such wide variance with those of the product of short periods of photosynthesis that it is very unlikely that there is an intimate connection between the dark "pick-up" product as we obtained it under either aerobic or anaerobic conditions and the "pick-up" which, it appears, must precede photosynthesis.

It is a disappointment to those of us who have sought to study photosynthetic dark fixation by tracer methods that all such studies (Ruben *et al.* (1), Benson and Calvin (2), Allen, Gest and Kamen (5), as well as the present work) can be interpreted quite adequately as additional demonstrations of so-called "respiratory fixation." No one has yet convincingly separated "photosynthetic" from "respiratory" dark fixation, and there seems little evidence to support the belief that the dark fixation products which have been detected by these studies bear a relation to photosynthesis.

Turning to the results obtained with illuminated algae, we see that the assimilated carbon appears first in fraction $B_{(\text{light})}$ and is subsequently transformed *photochemically* into fractions A and C. Thus, we are forced to consider the compound, $B_{(\text{light})}$, as an *intermediate* rather than as a *final product* of photosynthesis.

A comparison of the results of our kinetic studies of the formation and conversion of this intermediate with other facts known about photosynthesis suggests certain limitations on the number and kind of intermediates which photosynthesis may involve. The fact that the photosynthesizing cell rapidly distributes tracer carbon among divers

⁶ Note added in proof: Meanwhile a second article by Calvin and Benson has appeared [*Science* 107, 476 (1938)] with new experiments based on the procedure described in this paper. Despite the apparent duplication of method, their results are again in disagreement with ours.

compounds in several solubility fractions, and this *without any significant initial lag*, requires that *only one intermediate* be present in fraction B in large concentration. With respect to its content of tracer, short period $B_{(\text{light})}$ is, therefore, essentially homogeneous for, if it were not, curves of per cent tracer in B *vs.* time (Figs. 6 and 7) should be noticeably sigmoid; *i.e.*, the tracer would first accumulate in the B fraction, and an initial lag would precede its transformation into the other fractions. No such sigmoid character was manifest in data from any of our time studies, so we must conclude that no intermediate *in high concentration and stable under our conditions of extracting the cells* stands between carbon dioxide and $B_{(\text{light})}$ in the reaction sequence of photosynthesis.

The fact that practically all assimilated tracer is found in the B fraction after very brief intervals of photosynthesis (*i.e.*, the B curves of Figs. 6 and 7 extrapolate to 100% at zero time) could be explained by assuming that $B_{(\text{light})}$ is, indeed, the earliest compound in the reaction sequence which incorporates tracer during photosynthesis, namely, that it is the much discussed dark fixation product of carbon dioxide [*e.g.*, Aufdemgarten (6); McAlister (7), Ruben *et al.* (1), Franck and Herzfeld (8)]. But $B_{(\text{light})}$ is surprisingly stable in the unilluminated cell, whereas the known dark fixation products, such as oxaloacetic or oxalosuccinic acids readily take part in ordinary metabolic reactions and exchange carbon dioxide quite easily. However, the very low carbon dioxide concentration which suffices to saturate the photosynthetic mechanism requires that some dark fixation product stand between free carbon dioxide and the first photochemical reaction product. More than one reason may be suggested for our failure to find evidence for the existence of the dark pick-up product. It may be, chemically or thermally, an unstable substance which, under our methods of extraction, decomposes and loses the tracer as carbon dioxide. It may be stable but present in very low concentration relative to that of $B_{(\text{light})}$. The most interesting possibility is that the carbon dioxide acceptor in the dark pick-up reaction is itself a photochemical product. Thus, $B_{(\text{light})}$ perhaps combines in one molecule the properties of a first intermediate with those of a dark acceptor for carbon dioxide. The characteristics of $B_{(\text{light})}$ —retention of tracer in the B fraction in the dark, rapid transport into other fractions in the light, the influence of the presence of carbon dioxide on this transport, and the high concentration of tracer carbon in $B_{(\text{light})}$ at saturation—can be explained along lines

already developed in some detail by Lipmann (9). Carbon dioxide may be added to a substance produced by photosynthesis ($B_{(\text{light})}$) and there reduced. After reduction, this substance may repeat the process until a limiting molecular weight is reached, whereupon the molecule is broken down into fragments of lower molecular weight and the fragments are distributed to all the cell components. The portion remaining in fraction B adds tracer again and the cycle repeats. With such a mechanism, tracer added to photosynthesizing cells would begin to appear in fractions A and C without appreciable time lag, and $B_{(\text{light})}$ would increase in tracer content until completely derived from the added tracer. Illumination in the absence of carbon dioxide would necessitate a change in the manner of transformation of $B_{(\text{light})}$. $B_{(\text{light})}$ would be stable in the dark. At the present state of our knowledge, this can be advanced only as a reasonable hypothesis, not as an exclusive or proven mechanism.

SUMMARY

With radioactive C^{14} as a tracer, the fixation of carbon dioxide in the dark and its assimilation in the light was followed by manometric and chemical means in the alga *Scenedesmus*. Upon separation of the cellular constituents into 3 fractions, water-soluble, benzene-soluble, and insoluble, it was found that the water-soluble fraction, containing less than 2% of the dry weight of the algae, always had the greatest activity in terms of counts/mg. of sample. Kinetic studies showed that this fraction, ($B_{(\text{dark})}$ or $B_{(\text{light})}$), respectively, was the first to appear at the beginning of any fixation or assimilation period. $B_{(\text{dark})}$ can be formed under anaerobic conditions but, in the presence of air, it is rapidly respired and transformed into other compounds. $B_{(\text{light})}$ is stable against metabolic attack in the non-illuminated cell, but is transformed photochemically into the other fractions, slowly in the absence of carbon dioxide, rapidly in the presence of carbon dioxide. Kinetic studies, as well as the photosensitivity of $B_{(\text{light})}$, make it preferable to consider it as the first stable intermediate rather than as the first product of photosynthesis. A comparison between the rates and total amounts of light and dark fixation of tracer carbon dioxide and the properties of the substances resulting from these fixations provides no support for the existence in measurable quantity of a stable dark fixation product identifiable as a precursor to the photochemical reduction of carbon dioxide. It appears possible that $B_{(\text{light})}$ may combine in one molecule

the properties of an intermediate in the reduction process and those of a carbon dioxide acceptor. Those dark fixation products which have been isolated by our methods have either an indirect relation to photosynthesis or none at all.

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Isotopic Analysis of the Oxygen Evolved by Illuminated Chloroplasts in Normal Water and in Water Enriched with O¹⁸

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INTRODUCTION

The observation of the photochemical evolution of oxygen by illuminated chloroplasts suspended in a solution of an oxidizing agent has been recently extended from the original findings of Hill and Scarisbrick (1,2,3) to include such oxidants as ferricyanide, *p*-benzoquinone, naphthoquinones, chromate, and several oxidation-reduction indicators (4,5,6,7,8). The stoichiometry of these reactions, except in the case of chromate, indicates the splitting of water into molecular oxygen, while the hydrogen of the water effects reduction of the oxidant. The possible connection between this photochemical reaction and photosynthesis has been adequately discussed in the papers of Hill and of Warburg (1,2,5) and was treated in a review by French (9). Previous investigations by Ruben *et al.* (10), by Dole and Jenks (11) and by Vinogradov and Teis (12,13) have shown that oxygen evolved in photosynthesis is derived from water, while Yosida *et al.* (14) report two-thirds originates from water and one-third from carbon dioxide. All these investigations were done using either water enriched in O¹⁸ or by taking advantage of the difference in the densities of fresh water and water made from the oxygen of carbonate compounds, which contain a higher concentration of O¹⁸. We have undertaken similar investigations to determine if the oxygen evolved by isolated chloroplasts is derived from water as would be expected if the reaction involves the oxygen evolution step of photosynthesis.

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METHODS

Chloroplasts were isolated from leaves of spinach (*Spinacea oleracea*) obtained from a local market or from Swiss chard (*Beta vulgaris*) grown in a greenhouse. An outline of the procedures used, with attention to details given later, is as follows:

(a) Collection of the oxygen evolved by the illuminated chloroplasts suspended in a solution of the oxidant.

(b) Collection of the oxygen produced by electrolysis of the water obtained by distilling, or by lyophilizing, the same chloroplast-oxidant mixture used in (a).

(c) Measurement of the molecular ratios, $O^{18}O^{16}/O^{16}O^{16}$, of the samples obtained under (a) and (b).

1. Collection of the Oxygen Evolved by the Illuminated Chloroplasts

The apparatus used and its dimensions are given in Fig. 1. It consists essentially of a glass reaction chamber about 2 cm. thick with flat parallel sides and sidearm. The bottom of the chamber is attached to a mercury reservoir. At the top a T can be inserted which holds the sample bulb and to which a vacuum can be applied.

A weighed amount of the oxidant to be used was dissolved in 25 ml. of the isotopically enriched water, to which was added 1 ml. of 1 *M* phosphate buffer, pH 6.5. Before introducing this solution into the vessel, stopcock 1 was closed, 2, 3, and 4

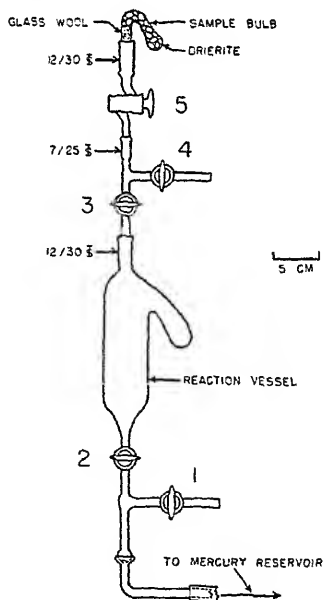


FIG. 1. The reaction vessel used to produce and collect oxygen from the photochemical action of illuminated chloroplasts suspended in solutions of several oxidants.

opened, and a vacuum from a Hyvac pump applied through stopcock 4. At the same time the mercury reservoir was lowered and adjusted so that the mercury level in the vessel was below stopcock 1. The pressure tubing connecting the reservoir and the vessel was then shaken to remove any entrapped air bubbles. The level of the mercury was then raised to just fill the capillary of stopcock 2, which was then closed. The vacuum was removed, the oxidant-buffer solution pipetted into the reaction chamber, and from 2 to 4 ml. of the chloroplast suspension introduced into the sidearm. The solution and suspension were degassed by boiling under vacuum at 10°C. for approximately 15 min. To prevent loss of the enriched water during the boiling process, a trap immersed in a dry ice-acetone mixture was interposed between the pump and the vessel. After degassing, stopcock 3 was closed and the chloroplasts tipped into the solution of the oxidizing agent. The mixture was illuminated from both sides for 45 min.

The illumination system consisted on one side of a 1000 w. tungsten bulb with a parabolic reflector, a 4" water filter, and a Corning No. 348 red filter which transmits light of wavelengths longer than 5700 Å. On the other side was a No. 2 G. E. Mazda Photoflood lamp, with a similar reflector and filter system. The reaction mixture was kept at 16°C. by streams of water flowing down each face of the vessel.

During the illumination period the space above stopcock 3 and the sample bulb were evacuated to approximately 0.5 mm. of mercury or less. After the illumination period, stopcocks 4 and 5 were closed and stopcock 2 opened, causing the mercury to push the mixture to the top of the vessel. The volume of gas obtained usually was between 0.5 and 0.8 ml. Stopcock 3 was opened, allowing the liquid to rise into the T tube. Stopcock 5 of the sample bulb was opened slowly and the gas collected. The sample bulb was of approximately 10 ml. capacity and was filled with Drierite, CaSO_4 anhyd. (Mesh No. 8).

2. *Electrolysis*

The design and dimensions of the combined distillation-electrolysis apparatus are given in Fig. 2. The reaction mixture in the Kjeldahl flask was degassed by boiling under vacuum, and the entire system, including the condenser, collection chamber and electrolysis cell, evacuated through stopcock 2. Stopcocks 1 and 2 were closed and approximately 12–15 ml. of the water distilled into the collection chamber by gently heating the Kjeldahl flask. The vacuum in the electrolysis cell was released, the T removed, and approximately 0.1 g. of anhydrous sodium sulfate placed over one of the electrodes. With stopcocks 1, 3, and 6 closed, the electrolysis cell was evacuated through stopcock 5 to less than 0.05 mm. of mercury. Stopcock 4 was closed and 1 opened, the water from the collection chamber being forced into the cell by its own vapor pressure. When a sufficient volume had flowed into the cell, stopcock 1 was closed and 3 opened. This forced the solution into the positive side of the cell to stopcock 4. The sample bulb was evacuated and 1–1.5 ml. of oxygen collected by alternately opening and closing stopcocks 4 and 6 during electrolysis by direct current. The current through the solution was approximately 0.5 amp.

3. *Determination of the $\text{O}^{18}\text{O}^{16}/\text{O}^{16}\text{O}^{16}$ Ratios of the Samples*

The ratios were determined by a mass spectrometer recently constructed by Dr. A. O. Nier of the Department of Physics, University of Minnesota (15). Samples from

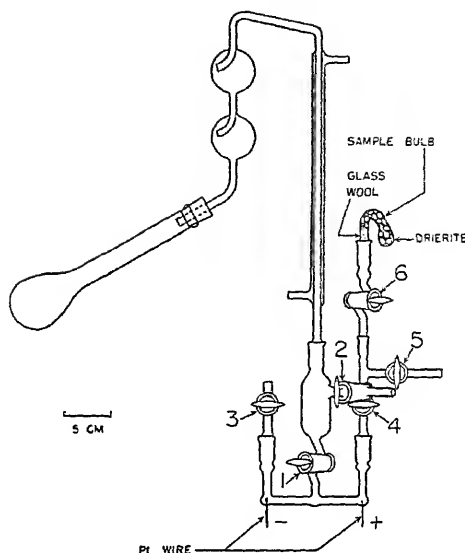


FIG. 2. The apparatus used to distill and electrolyze the water which had been used in the photochemical experiments. Oxygen was collected in the sample bulb.

a tank of commercial oxygen were used as the standards to make the results obtained on different days comparable to each other.

RESULTS

The ratios obtained using ferricyanide, *p*-benzoquinone, phenol indophenol, 2,6-dichlorophenol indophenol and chromate, are given in Table I. Exp. 1 and 2 were made with the same stock solution of ferricyanide prepared from laboratory distilled water. They served as checks on the reproducibility of the procedures used, and showed them to be fully satisfactory for the purpose of the investigation. The results using the enriched water (Exp. 3-8) show that water is the source of the oxygen evolved by the illuminated chloroplasts. Although the data obtained in Exp. 6 and 8 do not show as close agreement with the electrolysis samples as the previous ones, they do show that the ratio for the evolved oxygen is considerably higher than that for electrolyzed unenriched laboratory distilled water. In these cases the divergences were probably due to an analytical error with the mass spectrograph resulting from the necessity of using the considerably smaller volumes

TABLE I
*Isotopic Composition of Oxygen Evolved by Illuminated Chloroplasts
 as Compared with that of the Suspending Water*

Exp.	Water used	Oxidant	Ratio $O^{18}O^{16}/O^{16}O^{16} \times 100$	
			Method of O_2 production	
			Photo-chemical	Electrolysis
1	Normal	0.02 M ferricyanide	0.39	0.38
2	Normal	0.02 M ferricyanide	0.39	0.38
3	Enriched	0.02 M ferricyanide	1.4	1.3
4	Enriched	0.02 M ferricyanide	0.84	0.83
5	Enriched	0.0067 M quinone	0.62	0.61
6	Enriched	0.0035 M indophenol	0.57	0.62
7	Enriched	Dil. 2,6-dichlorophenol indophenol	0.54	(0.62-0.49)
8	Enriched	0.00221 M K_2CrO_4	0.52	0.49

of gas evolved by the chloroplasts suspended in these oxidants. In the case of the blue dye 2,6-dichlorophenol indophenol, it was necessary that its concentration in the reaction vessel at any time during the experiment be low enough to prevent its totally absorbing the light. This was accomplished by inserting below stopcock 2 a reservoir containing dye dissolved in the enriched water-buffer mixture, and admitting small amounts at a time. The control obtained by electrolysis of this sample was lost, but its composition can be approximated as being between those obtained in Exp. 6 and 8. With chromate, pH 8.3 KCl-borate buffer was used to take advantage of the fact that the exchange of oxygen between chromate and water is retarded in alkaline solution (15).

DISCUSSION

It is evident from the data given in Table I that the oxygen evolved by illuminated chloroplasts is originally derived from water, but these data alone do not afford definite proof that the oxygen resulted from splitting of water brought about by a photochemical reaction. The stoichiometry of the Hill reaction, and of the reactions with quinone and phenol indophenol indicate that such is the case. The question remains, however, as to whether the evolved oxygen results from a

photodecomposition of some substances stored within the chloroplasts, which may have exchanged its oxygen with that in the water.

Chlorophylls a and b have been found to absorb oxygen (17, 18), but only when dissolved in organic solvents such as ethanol, and no evidence has been found that the process is reversible (19). That the reverse of such a reaction is not occurring here is shown by the fact that Conant *et al.* (17) and Fischer and Riedmair (18) found only one mole of oxygen absorbed per mole of chlorophyll, which is in contrast to the results of many experiments carried out by the writers in which the ratio of the moles of oxygen evolved to the moles of chlorophyll present in active chloroplasts has been as high as 20.

Carotenoids are known to be readily autoxidizable and when exposed to air for a period of several weeks have absorbed even more than 6 moles of oxygen per mole of carotenoid (ref. 19, p. 474). Baur (20) has reported a reversible binding of oxygen by chloroform solutions of α -carotene treated with ultraviolet radiation. However, by the same reasoning as used with respect to chlorophyll, a reversible binding of oxygen by the carotenoids present in the chloroplasts will not account for the volumes of oxygen found by extended manometric determinations. The fact that intact cells of *Chlorella* evolve oxygen in the presence of Hill's solution (7) or quinone (5) also shows that oxygen evolution by isolated chloroplasts does not result from a photodecomposition of a substance formed during the isolation procedure.

If the oxygen had been evolved from a stored, oxygen-containing substance, a very high rate of oxygen exchange between it and the isotopically enriched water would have had to occur for the compositions of the photochemical and the electrolysis samples to have been as close as they were. The data (Exp. 1-5, 8) are such, however, that, instead of the isotopic ratio for the oxygen evolved by the chloroplasts being lower than that of the electrolysis sample, which would have indicated an exchange, it was actually slightly higher. Whether this increase is due to the techniques used or indicates an actual fractionation by the chloroplasts was not determined.

ACKNOWLEDGMENT

The writers wish to thank Dr. John Tate and the Rockefeller Foundation for meeting the expenses involved for the materials and apparatus used, Dr. A. O. Nier for the supply of isotopically enriched water and for his frequent advice, and Mrs. R. C. Boe for the analyses of the isotopic compositions of the samples.

SUMMARY

We believe that one can conclude that the oxygen evolved by illuminated chloroplasts in the presence of various oxidants originates from the splitting of water molecules brought about by a photochemical reaction.

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The Metabolism of Ether Acids ¹

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INTRODUCTION

The high water-solubility of the calcium salts of ether acids led us to try the salts as calcium therapeutics. The use of the salts as medicinals will be reported later. In this paper we present data on the fate in the rabbit of three ether acids that may eventually prove to be useful as solubilizing ions.

The ether linkage did not block metabolism of the acids. This was contrary to what we expected, since Levey and Lewis (1) found that the phenoxy group did stop any further utilization of a carbon chain beyond the last two carbon atoms. From the ingestion of 1 g. samples of calcium ethoxyacetate we recovered around 40% in the urine and none in the feces. With methoxyacetate and α -methoxypropionate the recoveries were even lower.

As yet, we cannot say what happens to the ether acid unaccounted for. The acid might be stored, hydrolyzed to glycolic acid, or oxidized directly. Oxidation seemed the most likely fate, and we were able to show that liver extracts slowly oxidized ethoxyacetic and methoxyacetic acids.

EXPERIMENTAL

Ether Acids

Ether acids were made by the method given for ethoxyacetic acid (2). Since the usual laboratory and commercial preparations contain unreacted monochloroacetic acid as an impurity, this must be removed by boiling with an excess of sodium hydroxide solution, acidifying and extracting with ether, then drying and distilling under vacuum.

¹ Presented at the Spring Meeting of the American Chemical Society, Chicago, 1948.

The calcium salts of the ether acids are exceptionally soluble in water and in 90% alcohol. They crystallize easily from water with two moles of water of hydration.

The rabbits were housed in metabolism cages and fed a stock pellet diet. For each experiment an amount of solution containing 1 g. of calcium salt (about 0.5 g./kg.) was given by stomach tube. The urine samples were collected every 24 hr. for 5 days.

Alkoxy Determinations

Ethoxyl and methoxyl determinations were performed according to the micro Zeisel method as described by Niederl and Niederl (3) with the following modifications. Five ml. of urine were evaporated to dryness without charring in a small dish on a sand bath. The residue was dissolved in phenol and acetic anhydride by warming (60–80°C.) and then transferred to the reaction flask, using additional liquid phenol as a wash. To complete the transfer the 2 ml. of HI used in the reaction were added to the dish in 1 ml. portions and transferred.

Control experiments, in which we added known amounts of calcium ethoxyacetate to rabbit urine, gave analytical recoveries of 98%. The blanks on 5 ml. of urine ran as an average 2.0 ml. of 0.03 *N* sodium thiosulfate. The variation in blanks (0.2–0.4 ml.) did not introduce a significant error, since the titration for 1.0 mg. of ethoxyacetic acid amounts to 1.91 ml. of 0.03 *N* thiosulfate.

RESULTS

First attempts to trace the fate of ethoxyacetic acid in rabbits led us to try isolating the acid from the urine after oral administration. We never succeeded in obtaining the acid or a derivative of the acid. Most of the difficulty was due to interference from hippuric acid that extracted with the ethoxyacetic acid on prolonged extraction and went through the separation procedure by way of the calcium salts. We further encountered a solid acid in the extracts that we did not identify.

We then turned to tracing the ethoxyacetic acid by determining its ethoxyl group directly on urine and feces. The ethoxyl thus obtained was not due to ethyl alcohol that might have split from the acid by hydrolysis, since a drying of the urine sample served to drive off any volatile compounds. The ethoxyl blank on 5 ml. of normal urine corresponds to slightly more than 1 mg. of ethoxyacetic acid but remains reasonably constant.

After an oral dose of 1 g. of the calcium ethoxyacetate, the urine was collected daily over a 5-day period and analyzed for ethoxyl content, but the total amount obtained after 5 days was only 43% of that given by mouth as the calcium ethoxyacetate. Table I shows the results of a typical run. The feces gave no increase in ethoxyl content above the normal amount, thus showing that absorption of the acid was complete.

TABLE I
Excretion of Ethoxyacetic Acid by Rabbits

	Days following administration					Excreted
	1	2	3	4	5	
Urine	<i>g.</i> 0.113	<i>g.</i> 0.148	<i>g.</i> 0.052	0	0	<i>per cent</i> 43
Feces	0	0	0	0	0	0

One g. of the calcium salt by stomach tube.

Following ethoxyacetic acid we administered methoxyacetic acid and *dl*- α -methoxypropionic acid (as the calcium salts). Both of these acids gave smaller recoveries in the urine than did ethoxyacetic. With methoxyacetic acid 25% was recovered; with *dl*- α -methoxypropionic the recovery was 35%.

Two routes of metabolism suggest themselves to account for the missing ether acids. The first of these is a hydrolysis to yield the corresponding alcohol and glycolic acid or lactic acid. Glycolic acid can be metabolized slowly by the kidney (4), but some of it should have appeared in the urine had the mechanism gone through the fission of the ether linkage. We never detected glycolic acid in the urine. The second pathway would be oxidation. Obviously the usual β -oxidation cannot take place since the oxygen atom is in the β position ($\text{CH}_3\text{OCH}_2\text{CO}_2\text{H}$). ω Oxidation might, then, be possible. In the case of methoxyacetic acid, ω oxidation should have yielded the bicarbonate of glycolic acid, $\text{HO}_2\text{COCH}_2\text{CO}_2\text{H}$, which in turn should have given glycolic acid. Thus, neither oxidative mechanism can explain satisfactorily the disappearance of the acids.

Results somewhat similar to this type of oxidation were recently reported by Mackenzie and others (5). They gave rats methionine with radioactive carbon in the methyl group and found that radioactive carbon dioxide escaped from the lungs in considerable amounts.

The splitting of simple alkyl ethers of glycolic acid contrasts with the stability of the phenyl ethers. With the phenoxyacetic acid Levey and Lewis (1) found complete resistance to splitting. This was also true of acids that would convert to phenoxyacetic by the β mechanism. The phenoxy groups, therefore, act as blocking groups, whereas we found that the ethoxy and methoxy groups do not. Thus, we believe that the

latter is the first instance recorded for the catabolism of an oxygen ether linkage.

That oxidation of the acids can take place was actually demonstrated. A phosphate extract of fresh beef liver gave positive tests for dehydrogenase activity by the Thunberg method with ethoxyacetate and methoxyacetate as substrates. The results were later confirmed on the Warburg apparatus. Methoxyacetate as substrate for a crude enzyme preparation gave an increase in oxygen consumption of about 9 times that of the control ($Q_{O_2} = 0.454$; pH 7.2, phosphate buffer; 37°C ., air). The purification of the enzyme from liver is the object of current research.

The rabbits seemed to tolerate the ether acids without ill effects. In fact, one rabbit receiving repeated doses over a period of 8 weeks increased in weight from 2 kg. to 4 kg. The urine showed no evidence of albumin, sugar, or increase in glucuronides, and the pH did not shift from its normal region of 8 to 9. On autopsy, the animals revealed no kidney or liver damage. Indeed, the only untoward effect noted was a distinct diuresis with ethoxyacetate, a reaction that was not shared by methoxyacetate and *dl*- α -methoxypropionate. Table II illustrates the differences in the secretion of urine for the 3 acids.

TABLE II
Urine Volume after Administration of Ether Acids as Calcium Salts

Days	Ethoxyacetic	Methoxyacetic	Methoxypropionic
	<i>ml.</i>	<i>ml.</i>	<i>ml.</i>
1	68	44	48
2	198	104	47
3	130	34	65
4	226	109	60
5	189	71	35
Average	162	72	50

Rabbit weight approx. 3 kg.; water *ad lib*.

SUMMARY

The 3 alkoxy acids, ethoxyacetic, methoxyacetic, and α -methoxypropionic, were metabolized by rabbits when given orally as the calcium salts. The percentages excreted after 1 g. doses were 43, 23, and 35, respectively, for the 3 acids.

Liver extracts oxidized methoxyacetic acid.

The 3 ether acids were easily tolerated by rabbits.

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The Inhibition by Amidone^{1,3} of Pyruvate and Succinate Oxidation by Rat Brain and the Reversal of the Inhibition by Boiled Yeast Extract²

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INTRODUCTION

In a previous investigation (1) it was found that amidone inhibited the glycolysis of glucose by rat brain but had no inhibitory effect on glycolysis of glycogen, fructose-6-phosphate, or hexose-1,6-diphosphate. It thus appeared that the drug inhibited the enzyme hexokinase. This inhibition by amidone could be reduced or reversed by boiled yeast extract. The factor in yeast responsible for this effect was shown to be adenosinetriphosphate (ATP).

In the present investigation it has been found that amidone also inhibits the oxidation of lactic, pyruvic, and succinic acids. This inhibition may be completely reversed by boiled yeast extract as was the case with the inhibition of glycolysis by amidone. It was at first thought ATP might be the factor here also, but such was not the case. Neither ATP, adenosine-5-phosphoric acid, cozymase, alloxazine adenine dinucleotide, cocarboxylase, nor glutathione had any effect in reducing the inhibition produced by amidone. The exact nature of this factor in yeast is not yet known but it was found to be thermostable and dialyzable. It is destroyed on heating with alkali but is quite resistant to boiling in dilute acid and may be extracted by organic solvents.

¹ Kindly supplied by the Mallinckrodt Co.

² Funds for carrying out this work were kindly supplied by the Mallinckrodt Chemical Works.

³ Amidone or methadone (2-dimethylamino-4,4-diphenylheptanone-5).

METHODS

Rat brain and kidney were homogenized in the buffers described and in the concentrations indicated in the tables.

Yeast extract was prepared by heating Fleischmann's bakers' yeast with an equal weight of water at 100°C. for 15 min., unless otherwise specified, and centrifuging. The supernatant fluid was used in the experiments described.

Oxygen consumption was measured in Warburg manometers of about 15 cc. capacity at 37°C. The gas phase was air.

In Thunberg experiments, the methylene blue was placed in the bulb, and tissue, substrate, drug and buffer in the main tube. The Thunberg tubes were then alter-

TABLE I

Effect of Amidone on Pyruvate Oxidation by Homogenized Rat Brain

Each Warburg vessel contained 150 mg. homogenized brain, 0.02 *M* pyruvate, 0.13 *M* NaCl, 0.002 *M* KCl, 0.03 *M* Na-K phosphate buffer,^a pH 7.4, in a final volume of 1.5 cc. When calcium was present, the concentration was 0.001 *M*. Temperature = 37°C.

Experiment	Duration of expt.	Conc. of amidone	mm. ³ O ₂ absorbed					
			Control		With drug			
			-Ca	+Ca	-Ca		+Ca	
						Per cent effect		Per cent effect
1	min. 90	<i>M</i> .00058	335	303	338	0	255	-16
		.00116			317	-5	135	-56
		.0023			214	-36	29	-91
2	60	.00058	232	240	212	-9	175	-27
		.00116			203	-13	119	-50
		.0023			127	-40	19	-89
3	90	.00116	274	348	226	-18	185	-47
		.0023			118	-57	65	-81
4	80	.00116	273	325	240	-12	221	-32
		.0023			140	-49	108	-67
5	75	.00116	331		264	-20		
		.0023			148	-55		

^a Na-K phosphate buffer, pH 7.4, contained equimolar solutions of Na₂HPO₄ and KH₂PO₄ in the proportions of 8 to 2, respectively.

TABLE II

Effect of Amidone on Succinoxidase

Each Warburg vessel contained 20 mg. homogenized kidney or 100 mg. homogenized brain, 0.05 *M* succinate, 26 γ CaCl_2 , 32 γ AlCl_3 , 0.7 or 1.0 cc 0.1 *M* Na-K phosphate buffer in a final volume of 2 cc. Temperature = 37°C.

Experiment	Duration of expt.	Tissue	Conc. of amidone	mm. ³ O ₂ absorbed		
				Control	With amidone	Per cent effect
6	min. 60	Brain	<i>M</i>	203		
			.00044		230	+13
			.00088		232	+14
			.00176		138	-32
7	60	Kidney	.00044	361	267	-26
			.00088		207	-43
			.00176		99	-73
8	60	Kidney	.00044	266	217	-18
			.00088		126	-53
			.00176		81	-70

nately evacuated and filled with nitrogen 2 or 3 times, and finally evacuated and closed. The tubes were equilibrated at 37°C. for 2 min. before mixing. The time for decolorization of methylene blue was measured and recorded.

RESULTS

The tables contain only a few experiments which are representative of a considerably larger series.

*Effect of Amidone on the Oxidation of Lactate,
Pyruvate, and Succinate*

Table I shows the effects of amidone in different concentrations on the oxidation of pyruvate by rat brain. In most experiments, the addition of calcium appeared to enhance the inhibition produced by amidone, although in a few experiments it had little effect. In the presence of added calcium the lowest concentration of amidone to produce a significant inhibition was 0.00058 *M*. Concentrations of 0.0023 *M* produced marked inhibitions. Table II shows the effects of amidone on succinoxidase of rat brain and of kidney. It may be seen that brain suc-

cinoxidase is less affected by amidone than is kidney succinoxidase under similar conditions. The kidney succinoxidase was inhibited to about the same extent as the pyruvate oxidizing system of brain. The media in these two sets of experiments were, however somewhat different.

TABLE III

*Inhibition by Amidone of Dehydrogenase Activity (Thunberg Technique)
and the Reduction of This Inhibition by Boiled Yeast Extract*

Thunberg tubes contained 0.5 cc. methylene blue (0.07%), 100 mg. brain or 20 mg. kidney, 0.03 *M* Na-K phosphate buffer, pH 7.4, and 0.02 *M* lactate or pyruvate or 0.05 *M* succinate. Final volume 3cc. Temperature 37°C. The experiments were carried out in duplicate or triplicate and the results averaged.

Experiment	Tissue	Substrate	Conc. of amidone	Yeast extract	Time for decolorization of methylene blue in minutes	
					Control	With amidone
9	Brain	Lactate	<i>M</i> .0023	—	10.6	23.3
10	Brain	—	—	—	37	
	Brain	Lactate	.00057	—	14	15
	Brain	Lactate	.00114	—		16.25
	Brain	Lactate	.0017	—		26
11	Brain	—	—	—	22	
	Brain	Pyruvate	.00114	—	8	9.5
	Brain	Pyruvate	.0017	—		11
12	Brain	Pyruvate	.0023	—	7	15
13	Brain	—	—	—	15.25	
	Brain	Lactate	.0023	—	5	19.5
	Brain	Lactate	.0023	0.8 cc. old	5.75	7.5
	Brain	Lactate	.0023	0.8 cc. fresh	6	8
14	Kidney	—	.0032	—	>180	>180
	Kidney	Succinate	.0032	—	8.5	17.5
	—	—	.0032	—		No change after 180
15	Kidney	Succinate	.0008	—	10.5	13
	Kidney	Succinate	.0016	—		14.5
	Kidney	Succinate	.0032	—		18.5
	Kidney	Succinate	.0032	0.7 cc.	7.5	8.5

TABLE IV

Effect of Boiled Yeast Extract and Certain Factors Present in Yeast on the Inhibition Produced by Amidone of Pyruvate and Succinate Oxidation

The experiments with brain and pyruvate as substrate were as described in Table I, those with kidney and succinate as described in Table II. P = pyruvate, S = succinate, E = boiled yeast extract.

Experiment	Duration of expt.	Tissue	Substrate	Conc. of amidone	Other additions	mm. ³ O ₂ absorbed		
						Control	With amidone	Per cent effect
16	70	Brain	P	.0023	—	365	205	-44
		Brain	P	.0023	0.4 cc. E 5 days old	362	509	+29
		Brain	P	.0023	0.4 cc. E fresh	401	430	+9
17	70	Brain	P	.0023	—	365	195	-47
		Brain	P	.0023	0.4 cc. E 100° 10'	366	342	-7
		Brain	P	.0023	0.4 cc. E 100° 30'	352	338	-4
		Brain	P	.0023	0.4 cc. E 100° 60'	328	330	0
18	70	Brain	P	.0023	—	337	184	-45
		Brain	P	.0023	0.4 cc. E evap. to dryness and redissolved	319	295	-8
		Brain	P	.0023	0.4 cc. E dialyzed—48 hr.	320	185	-42
19	60	Kidney	S	.00088	—	255	114	-55
		Kidney	S	.00088	0.6 cc. E	307	297	-3
20	60	Kidney	S	.0017	—	224	113	-50
		Kidney	S	.0017	0.6 cc. E	328	293	-11
21	75	Brain	P	.0023	—	358	198	-45
		Brain	P	.0023	ATP .0006 M	369	190	-49
		Brain	P	.0023	ATP .0012 M	338	172	-49
22	60	Brain	P	.0023	—	239	140	-41
		Brain	P	.0023	Muscle adenylic acid .0012 M	231	63	-73
		Brain	P	.0023	Muscle adenylic acid .0024 M	202	11	-95
23	60	Brain	P	.0023	—	254	120	-53
		Brain	P	.0023	Coccarboxylase .0005 M	255	133	-48
		Brain	P	.0023	Cozymase .0005 M	238	136	-43
24	85	Brain	P	.0023	—	570	298	-48
		Brain	P	.0023	Glutathione .002 M	587	297	-49

With methylene blue as hydrogen acceptor (Thunberg technique), the oxidation of lactate, pyruvate and succinate was inhibited by amidone in concentrations as low as 0.001 *M* (Table III). The oxidation of all 3 substrates appeared to be about equally effected.

*Effect of Boiled Yeast Extract on the Inhibition of Oxidation
Produced by Amidone*

The inhibition by amidone of oxidation of lactate, pyruvate or succinate by homogenized tissues could be reduced or completely reversed by boiled yeast extract (Tables III and IV).

In some cases, the addition of boiled yeast extract transformed an inhibition by amidone into an acceleration. In one experiment (No. 16) in Table IV, amidone inhibited pyruvate oxidation by 44%. Amidone with boiled extract produced an acceleration of oxidation of 29%.

The inhibition by amidone of methylene blue reduction was also reduced or reversed by yeast extract (Table III).

The factor in yeast producing this reversal of amidone action was thermostable since heating at 100°C. for 60 min. had no effect on its activity (Table IV, Experiment 17). The extract could also be evaporated to dryness on a water bath without change (Experiment 18). The molecular weight was apparently low, since the factor was lost on dialysis in a cellophane sac (Experiment 18). The extract remained active on standing for several days in the refrigerator (Experiment 16). None of the following compounds reduced the inhibition by amidone to any extent—ATP, cocarboxylase, cozymase, alloxazine adenine dinucleotide, nicotinamide, glutathione, or ascorbic acid. Adenosine-5-phosphoric acid likewise did not reduce the inhibition, but instead increased the inhibition produced by amidone to some extent.

DISCUSSION

We have previously shown that amidone differed from morphine and demerol⁴ in its effect on glycolysis by brain (1). Whereas morphine and demerol had no inhibitory effect, amidone in similar or lower concentrations inhibited glycolysis of glucose, presumably by blocking the enzyme hexokinase. This inhibitory effect could be reduced or completely reversed by ATP, depending on the concentration.

⁴ Ethyl-1-methyl-4-phenylpiperidine-4-carboxylate.

Quastel and Wheatley (2) have shown that morphine resembled the anaesthetics in inhibiting the oxidation of lactate and pyruvate by brain and in having no effect on the oxidation of succinate (3). We have confirmed these results. We have also found that demerol likewise inhibited the oxidation of pyruvate by brain but had no inhibitory effect on the oxidation of succinate (4). Neither morphine nor demerol had any inhibitory effect in Thunberg experiments in which methylene blue was the hydrogen acceptor, indicating that these drugs did not exert their inhibitory effect on the dehydrogenases (4).

Amidone, however, differed from morphine, demerol, and the anaesthetics, in that it inhibited the oxidation of pyruvate and of succinate, whether determined by measurement of oxygen consumption or of methylene blue reduction. Elliott *et al.* (5) have previously reported that amidone inhibited the oxidation of lactate and pyruvate, and Watts (6) found that it inhibited oxidation of succinate by brain. Since amidone inhibited the reduction of methylene blue in Thunberg experiments, with either lactate, pyruvate or succinate as substrate, it would seem that amidone may exert its inhibitory effect on the dehydrogenases. Amidone thus differs quite markedly from morphine, demerol, and the anaesthetics on brain metabolism. Robbins has observed that amidone, unlike morphine in equivalent doses, had minimal or no hypnotic effect when administered to patients (7).

The inhibition produced by amidone could be reduced or reversed by boiled yeast extract, when determined by either the Warburg or Thunberg techniques. In some cases amidone in the presence of boiled yeast extract accelerated oxidation by brain. This factor, present in yeast, was also found in certain boiled animal tissue extracts. None of the well known coenzymes in yeast gave this effect.

It was thermostable and dialyzable. It was destroyed by heating with dilute acid at 100°C. This factor is being further investigated.

SUMMARY

1. Amidone was found to inhibit the oxidation of pyruvate and succinate by animal tissues, whether determined by the Warburg or Thunberg techniques.
2. This inhibition produced by amidone could be reversed by a thermostable, dialyzable factor in boiled yeast extract, which did not appear to be any of the well known coenzymes.

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Protochlorophyll, Precursor of Chlorophyll

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INTRODUCTION

Seedlings grown in the dark contain small quantities of a green pigment called protochlorophyll. In the different mechanisms proposed for the greening of such etiolated seedlings, this pigment has been assigned various roles. Liro (1) considered it to be an artifact produced by the extraction of the etiolated leaves—an hypothesis discredited because of the identification of the characteristic absorption bands of protochlorophyll in the living etiolated leaf (2). Lubimenko (3) considered it to be a by-product of the chain of reactions leading to the formation of chlorophyll. But many other investigators, for example, Eyster (4), Rudolph (5), and Frank (6), have assumed it to be the precursor of chlorophyll.

The last of these assumptions has been based chiefly on the following observations: illumination of etiolated leaves causes a simultaneous disappearance of protochlorophyll and appearance of chlorophyll; and the action spectrum for the formation of chlorophyll (6) agreed with what was known at the time of the absorption spectrum of protochlorophyll from pumpkin seeds.

The correspondence of the action spectrum for the formation of chlorophyll and the absorption spectrum of protochlorophyll, especially the protochlorophyll of etiolated leaves, was uncertain for 3 reasons: the identity of leaf protochlorophyll with pumpkin seed protochlorophyll had never been established; the absorption spectrum of protochlorophyll from either source was inadequately known; and the "blue peak found in the effectiveness curve . . . [had] no exact counterpart in the report of the absorption properties of the pumpkin seed protochlorophyll molecule" (6).

Protochlorophyll has recently been isolated in pure form from etiolated barley seedlings and its absorption spectrum measured quantitatively (7). From the absorption curve it is obvious that the blue peak of the action spectrum curve has its counterpart in the absorption spectrum curve, a point which formerly had been in doubt. A comparison of the spectral absorption of protochlorophyll with the action spectrum for chlorophyll formation, as determined by Frank (6), is shown in Fig. 1. In general aspect, the two curves are similar. The absorption curve of

the protochlorophyll is, however, displaced to shorter wave lengths. This is not surprising, inasmuch as an analogous displacement occurs in the position of the long wavelength absorption band of protochlorophyll in ether solution and in the living leaf (2). When the absorption curve of protochlorophyll is shifted 11 $m\mu$ to longer wavelengths, it is

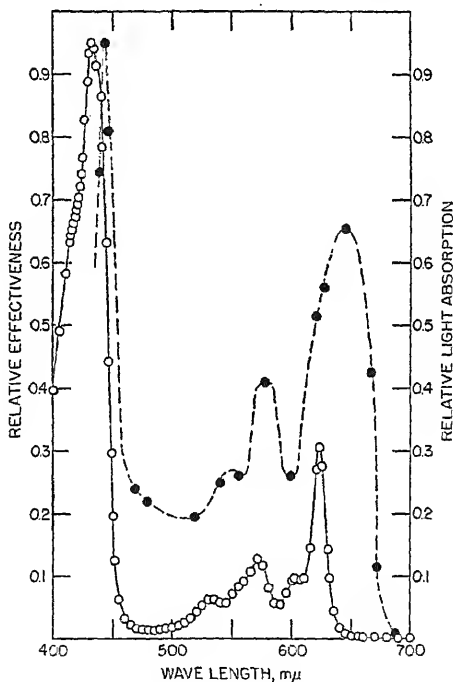


FIG. 1. The relative effectiveness curve (—●—) for chlorophyll formation (6) compared with the relative light absorption curve of protochlorophyll dissolved in ether (—○—). The relative light absorption curve of protochlorophyll has been adjusted so that the height of the chief absorption band equals the height of the corresponding band in the relative effectiveness curve.

remarkable how closely the wavelengths of maxima of the 2 curves correspond. Because of this correspondence it seems justifiable to conclude that protochlorophyll is the light-absorbing agent which is active in the initial production of chlorophyll.

The correspondence of the absorption spectrum of protochlorophyll and the action spectrum for chlorophyll formation does not prove, however, that protochlorophyll is transformed into chlorophyll by

photochemical action. Protochlorophyll may act only as a photochemical sensitizer for the reaction. But evidence has now been obtained from experiments on the formation of chlorophyll in etiolated barley seedlings at low temperatures which points to protochlorophyll as being the precursor of chlorophyll.

EXPERIMENTAL

In general, the methods described in the previous paper (8) have been used in this investigation for the growing and harvesting of the barley leaf material, for the extraction of the plant material, for the preparation of solutions, and for the spectrophotometric analyses.

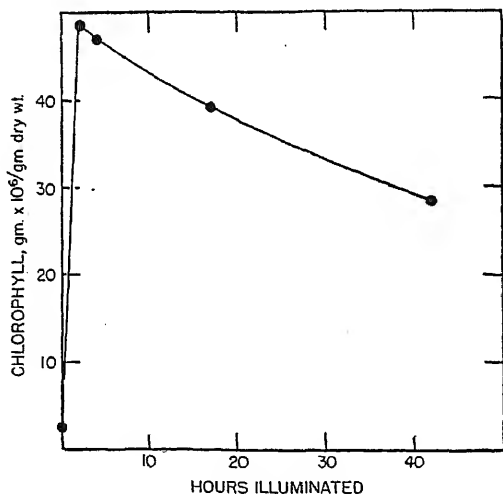


FIG. 2. Formation of chlorophyll in etiolated barley seedlings at 0°C.

The experiments on chlorophyll formation at 0°C. were performed as follows: A pot of seedlings was transferred from the dark cabinet, kept at room temperature, to the darkened cold cabinet maintained at $0 \pm 1.5^\circ\text{C}$. by means of a mechanical refrigeration unit. After the plants had been stored at this temperature in the dark for 2 hr., they were illuminated for the desired period by a 100-watt Madza lamp hung 50 cm. above the pot. The quantities of chlorophyll formed under these conditions and during various periods of illumination are shown in Fig. 2.

The results recorded in Fig. 3 were obtained from experiments performed under 3 different sets of conditions. The results represented by open circles were obtained at $0 \pm 1.5^\circ\text{C}$. under the conditions described in the previous paragraph. The results plotted with solid circles were obtained by illumination of seedlings contained in an

open glass jar which was placed in a tub of cracked ice. A temperature gradient existed inside the jar with a maximum temperature at the top of the jar of about 6 or 7°C. Illumination was furnished by a 100-watt Mazda lamp hung 40 cm. above the pot. The result plotted by the half-filled circle was obtained from barley seedlings illuminated at $4.5 \pm 0.3^\circ\text{C}$. A bank of six 15-watt fluorescent lamps hung 49 cm. above the pot containing the seedlings was used for the illumination. The experiment was carried out in a cold room in which air was vigorously circulated. (The author is indebted to Miss V. M. Koski for obtaining this last result.) In each case, comparison of the illuminated and unilluminated seedlings was made with seedlings from the same planting.

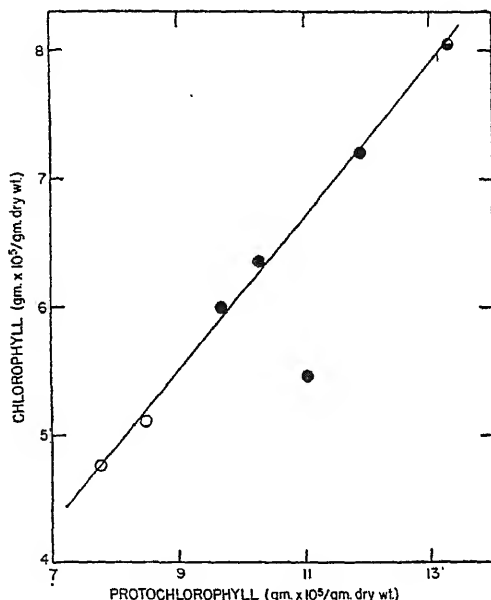


FIG. 3. The relation of the quantity of chlorophyll formed in barley seedlings by illumination for 2 hrs. at low temperatures to the quantity of protochlorophyll initially present in the etiolated leaves. Temperatures: ○ 0°C.; ◐ 4.5°C.; ● below 6 or 7°C. (ice bath).

The quantities of pigments recorded in Fig. 3 were calculated from light absorption data. The quantities of protochlorophyll were estimated from the absorption of the crude ether extracts at wave length 625 $m\mu$, assuming the specific absorption coefficient of protochlorophyll at this wavelength to be 32.86, the value obtained by Koski and Smith (7). The quantities of chlorophyll were calculated from the light absorbed at 662.5 $m\mu$ by the crude ether extract of leaves which had been illuminated for 2 hr. at low temperatures. This was the wave length at which the extract showed maximum absorption in the red end of the spectrum. The specific absorption coefficient was

taken to be 102.1, the value given by Zscheile and Comar (9) for the maximum absorption of chlorophyll *a* in this region of the spectrum. The assumption that only chlorophyll *a* is present is warranted by the observation of Goodwin and Owens (10) that in the beginning of greening "virtually no chlorophyll *b* is formed in oat plants," a finding which separate experiments of ours on barley plants substantiate. All values are referred to 1 g. of dry weight.

RESULTS AND DISCUSSION

When the seedlings of some plants are illuminated below certain critical temperatures they produce only limited quantities of chlorophyll but never turn green (3,11). This observation indicates that a limited amount of the precursor of chlorophyll is initially present in such leaves and is transformed into chlorophyll by illumination, but that the precursor is not regenerated by metabolic processes at the lower temperatures. The results shown in Fig. 2 indicate that this is true for etiolated barley seedlings when they are illuminated at 0°C. These results show also that the chlorophyll which is first formed during the first 2 hr. of illumination is gradually destroyed by further illumination.

If the limited quantity of precursor initially present in the barley seedlings alone is transformed to chlorophyll by illumination, then the amount of chlorophyll formed should be proportional to the amount of this precursor. And if protochlorophyll is this precursor, then the amount of chlorophyll formed should be proportional to the quantity of protochlorophyll initially present in the leaves.

A comparison of the quantities of chlorophyll formed at low temperatures during a 2 hr. period of illumination with the quantities of protochlorophyll initially present in the leaves shows that a direct proportionality exists between the two (Fig. 3). On a molecular basis, this comparison indicates that approximately 0.6 of a molecule of chlorophyll is formed for each molecule of protochlorophyll initially present in the leaves. Although unequal quantities of protochlorophyll were obtained from etiolated barley leaves from different plantings, the conditions by means of which the protochlorophyll content can be varied at will are not known.

To get a more reliable figure for the conversion of protochlorophyll to chlorophyll, certain corrections must be applied to the data just cited: one, to make allowance for any of the protochlorophyll initially present in the leaves which was not transformed; and the other, to

allow for the chlorophyll which was destroyed by the 2 hr. illumination. In two experiments where sufficient data were available to make allowances for these factors, it was found that 0.8 and 0.9 of a molecule of chlorophyll appear for each molecule of protochlorophyll which disappears. This transformation is remarkably quantitative for a biological process.

SUMMARY

In summary, it may be said that the evidence obtained, namely, the correspondence of the action spectrum for chlorophyll formation with the absorption spectrum of protochlorophyll, the direct proportionality of the amount of chlorophyll formed at low temperatures to the amount of protochlorophyll initially present in the etiolated leaves, and the near equivalence of the number of moles of protochlorophyll disappearing and the number of moles of chlorophyll simultaneously appearing, leaves little room for doubt that protochlorophyll is the precursor of chlorophyll in the early stages of greening of etiolated leaves.

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Addition Products of Dehydropeptides

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INTRODUCTION

The unsaturated character of the dehydropeptides, $\text{RCHCON} = \text{C}(\text{CH}_2\text{R}')\text{COOH} \rightleftharpoons \text{RCHCONHC}(= \text{CHR}')\text{COOH}$, is revealed in part by several types of addition reactions, such as the decolorization of bromine and permanganate (1-4), and the addition of catalytic hydrogen (1-4), of mercaptans (5,6), of amides (7), and of amines (8). The ease and mode of addition may, however, vary. Thus, although catalytic hydrogen and mercaptans add equally well to peptides of dehydroalanine and dehydrophenylalanine, acetamide adds only to acetyldehydroalanine but not to acetyldehydrophenylalanine (7). Furthermore, although mercaptans (5,6) and amines (8) add to acetyldehydroalanine to form, respectively, α -acylamino- β -arylmercaptopropionic acids, and α -acylamino- β -alkylaminopropionic acids, acetamide adds in such a way as to form chiefly α, α -di(acetamino)propionic acid (7).

Because of the potential biological significance inherent in the chemical reactivity of the α, β double bond in the dehydropeptides, we have studied the interaction of various reagents such as bromine, mercaptans and amines with 4 dehydropeptides, namely, acetyldehydroalanine (1), chloroacetyldehydroalanine (9), acetyldehydrovaline (10), and acetyldehydrophenylalanine (2). We have noted that (a) bromine reacts nearly stoichiometrically with acetyldehydroalanine and chloroacetyldehydroalanine, less than stoichiometric with acetyldehydrovaline, and not at all with acetyldehydrophenylalanine, (b) aryl mercaptans readily add to acetyldehydroalanine and acetyldehydrophenylalanine, but apparently not to chloroacetyldehydroalanine or acetyldehydrovaline, and (c) amines add only to acetyldehydroalanine and chloroacetyldehydroalanine (8), but apparently not to acetyldehydrovaline

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or acetyldehydrophenylalanine. The principal criterion used in following the addition or lack of addition of the reagents is the change or lack of change of the original spectrum of the dehydropeptide in the ultraviolet (8-11).

EXPERIMENTAL

Addition of Bromine

Bergmann and coworkers had shown that acetyldehydroalanine (1) and chloroacetyldehydroalanine (1) decolorized aqueous solutions of bromine, but they did not study the reaction quantitatively. We have employed 2 general procedures to follow the addition of bromine to dehydropeptides, namely, (a) by the decrease in specific absorption of certain of these compounds in the ultraviolet, and (b) by titration with standard thiosulfate.

Successive aliquots of stock solutions of the dehydropeptides in absolute alcohol were treated with varying quantities of a standardized solution of bromine in absolute alcohol. The absorption at the 2400 Å maximum was measured for acetyldehydroalanine and chloroacetyldehydroalanine and the changes portrayed in Fig. 1. The

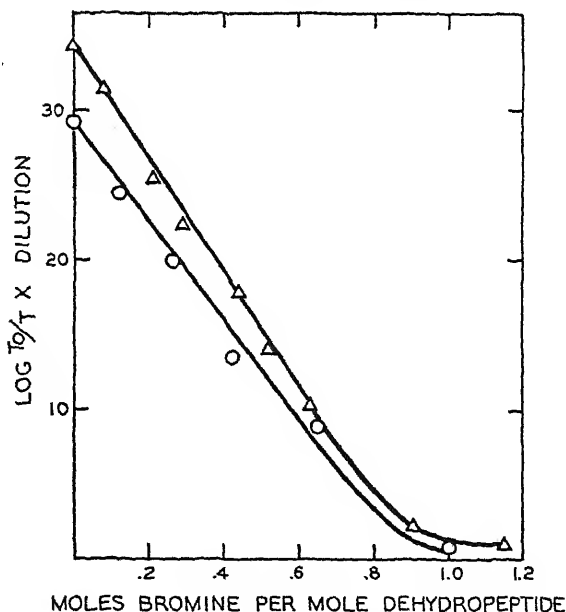


FIG. 1. Decrease in absorption at 2400Å of alcoholic solutions of acetyldehydroalanine O, and of chloroacetyldehydroalanine Δ, treated with increasing quantities of bromine. Ordinate refers to product of log of ratio of transmission by the dilution with bromine solution.

decrease in the absorption at this wavelength was proportional to the bromine added, and with a ratio of one mole of bromine to one of dehydropeptide, the absorption had practically disappeared. Acetyldehydrovaline could not be studied by this spectrophotometric titration because its spectrum reveals no maximum absorption (10), the slope of the absorption curve being too steep for accurate measurement of small changes in absorption at any wavelength between 2900 Å and 2200 Å. In the case of acetyldehydrophenylalanine, no bromine was added under the experimental conditions used, as shown by an unaltered absorption curve in the ultraviolet.

When an excess of bromine in glacial acetic acid solution was added to $8.5 \times 10^{-3} M$ solutions of the dehydropeptides in water and allowed to stand in the dark for 0.5 hr., treated with 15% KI solution, and then titrated with 0.1 *N* thiosulfate, it was noted that, in terms of moles of bromine absorbed per mole of compound, the value for acetyldehydroalanine was 0.81, for chloroacetyldehydroalanine 0.80, for acetyldehydrovaline 0.68, and for acetyldehydrophenylalanine 0.0. In contrast to the aliphatic dehydropeptides, acetyldehydrophenylalanine does not apparently react with bromine, a phenomenon presumably due to the increased aromatic character of the double bond conjugated to the benzene ring.

Attempts were made to isolate the acetyldehydroalanine-bromine addition product, but the compound was extremely unstable and broke down rapidly in the presence of water to yield free ammonia.

Addition of Mercaptans

The problem of the addition of mercaptans to the double bond of dehydropeptides possesses a special interest in view of the possibility that amino-acrylic acid and hydrogen sulfide may be intermediate products in the metabolism of cystine (and cysteine) (5,12). Mercaptans readily add to α , β -unsaturated ketones (13,14) and esters (13,15). Addition products of mercaptans with azlactones (13-19), unsaturated aldehydes (20-23), and dehydropeptides (5,6,19) have been employed as starting materials for the synthesis of sulfur-containing amino acids. For any of these addition reactions to take place, strongly basic or peroxide catalysts must be present.

We have refluxed 3 g. each of acetyldehydroalanine and acetyldehydrophenylalanine with 50 g. of either benzyl or phenyl mercaptan in the presence of 0.2 cc. of piperidine and just enough absolute alcohol to bring the reactants into solution. Anhydrous conditions were maintained. After 8-10 hr., the alcohol and excess mercaptan were removed by distillation *in vacuo*, the oily residue taken up in benzene and treated with an excess of petroleum ether. The solid material which separated was treated again in this fashion. and finally crystallized from the appropriate solvent.

DL-N-Acetyl-S-Benzylcysteine

From acetyldehydroalanine and benzyl mercaptan. Yield 2.4 g. (41%). Recrystallized from absolute alcohol as prisms. Product has properties described by Stekol (24). M.p.² 155-157°C. Calculated: C, 56.9; H, 5.9; N, 5.5; S, 12.7; found: C, 57.1; H, 5.8; N, 5.4; S, 12.7.

² All m.p. uncorrected.

DL-N-Acetyl-S-Benzyl-β-Phenylcysteine

From acetyldehydrophenylalanine and benzyl mercaptan. Yield 1.0 g. (20%). Recrystallized from a mixture of ethyl acetate and dry ether as prisms. M. p. 154–156°C. Calculated: C, 66.7; H, 5.8; N, 4.3; S, 9.7; found: C, 66.5; H, 5.7; N, 4.6; S, 9.6.

DL-N-Acetyl-S-Phenylcysteine

From acetyldehydroalanine and phenyl mercaptan. Yield 1.8 g. (33%). Recrystallized from aqueous alcohol as needles. M.p. 121–122°C. Calculated: C, 55.2; H, 5.5; N, 5.9; S, 13.4; found: C, 55.5; H, 5.3; N, 6.0; S, 13.2. The L-form of this compound, m.p. 142–143°C. has been described (25).

DL-N-Acetyl-S-Phenyl-β-Phenylcysteine

From acetyldehydrophenylalanine and phenyl mercaptan. Yield 1.9 g. (40%). Recrystallized as needles from ethyl acetate. M.p. 125–127°C. Calculated: C, 64.8; H, 5.4; N, 4.4; S, 10.2; found: C, 64.8; H, 5.9; N, 4.8; S, 10.0.

Attempts to use methyl and ethyl mercaptans were unsuccessful. No definite addition products could be obtained by the interaction of benzyl or phenyl mercaptan with either chloroacetyldehydroalanine or acetyldehydrovaline.

When incubated with rat liver extracts (8,9), no ammonia was evolved in 2 hr. from the mercaptan-substituted peptides. Whether the peptide bond was hydrolyzed was not investigated at this time. The loss in susceptibility of the acetyldehydroalanine addition product is another proof of the disappearance of the dehydropeptide character of the starting material.

Addition of Amines

The addition of primary and secondary amines to acetylenic ketones has been one of the methods of preparation of amino ketones (26). The addition of amines to acrylic acid esters leads to a number of products, including substituted β-aminopropionic acids (27). The ease of addition of amines to the double bond of chloroacetyldehydroalanine, accompanied by replacement of halogen by amine, and by loss of the specific absorption in the ultraviolet, has been noted (8).

We have attempted to prepare a number of substituted α-β diaminopropionic acids by addition of one mole of amine to one mole of dehydropeptide, using methylamine, dimethylamine, and benzylamine. When the aromatic amine was used, the procedure was to add amine in 10:1 ratio to the hot, alcoholic solution of the dehydropeptide and to boil under the reflux for 4–6 hr. On chilling, the products derived from acetyldehydrovaline and from acetyldehydrophenylalanine crystallized out, and were washed with ether and recrystallized. The products derived from acetyldehydroalanine were obtained as oily residues after alcohol and excess amine were

removed by distillation under reduced pressure. These residues could be brought to crystallization by treatment with acetone. When the aliphatic amines were used, the procedure was to dissolve the dehydropeptides in aqueous solutions of the amines (3–5 g. of peptide in 50 cc. of approximately 30% solutions of the amines), and to allow the mixtures to stand at 40°C. for 72 hr. After removal of the excess amine *in vacuo*, the residues could be brought to crystallization by treatment with either acetone or absolute alcohol.

α -Acetamino- β -Benzylaminopropionic Acid

From acetyldehydroalanine and benzylamine. Yield 21%. Recrystallized from absolute alcohol. M.p. 171–172°C. Calculated: C, 61.0; H, 6.8; N, 11.9; found: C, 60.8; H, 6.6; N, 12.2.

α -Acetamino- β -Dimethylaminopropionic Acid

From acetyldehydroalanine and dimethylamine. Yield 26%. Recrystallized as needles from methyl alcohol-dry ether. M.p. 178–179°C. Calculated: C, 48.3; H, 8.1; N, 16.1; found: 47.9; H, 7.6; N, 15.5.

α -Acetamino- β -Methylaminopropionic Acid

From acetyldehydroalanine and methylamine. Yield 24%. Recrystallized as prisms from methyl alcohol-dry ether. M.p. 164°C. Calculated: C, 45.0; H, 7.5; N, 17.5; found: C, 44.8; H, 7.1; N, 17.5.

When incubated with rat liver extracts (8), none of these compounds yielded ammonia, furnishing another proof of the loss of the dehydropeptide character of the starting material.

The products obtained by the interaction of methylamine, dimethylamine, and benzylamine with acetyldehydrophenylalanine and acetyldehydrovaline were all crystalline, stable, and gave elemental analyses which checked closely with those of compounds made up of one mole of dehydropeptide and one mole of amine (Table I). Spectrophotometric studies of the compounds in the ultraviolet, however, revealed absorption spectra practically identical with those of the dehydropeptide employed. Moreover, aqueous solutions of the amine products with acetyldehydrovaline reduced added bromine. These phenomena indicate that the compounds are most probably the amine salts of the otherwise unaltered dehydropeptides.

The molar extinction of the addition products of acetyldehydroalanine and acetyldehydrophenylalanine with mercaptans and with amines is given in Table II.

TABLE I

Amine Salts of Acetyldehydrophenylalanine and Acetyldehydrovaline

Analytical results							Spectra (in 80% alc.)		
Dehydropeptide	Amine		C	H	N	Recryst. from	Wave length ^a	ϵ	Melting points ^d
Acetyldehydrophenylalanine	Benzyl-amine	Found Calc.	69.4 69.2	5.8 6.4	9.1 8.9	Ethyl acetate	2700 ^a	13,500 ^b	196
Acetyldehydrophenylalanine	Dimethyl-amine	Found Calc.	61.1 61.0	6.4 6.7	11.7 11.8	Methyl alc.-ether	2800 ^a	16,500	153
Acetyldehydrophenylalanine	Methyl-amine	Found Calc.	62.4 62.4	7.7 7.2	11.1 11.2	Methyl alc.-ether	2800 ^a	13,500	183
Acetyldehydrovaline	Benzyl-amine	Found Calc.	63.5 63.6	7.2 7.5	10.6 10.6	Methyl alc.-ether	2400	5,600 ^c	173
Acetyldehydrovaline	Dimethyl-amine	Found Calc.	53.6 53.4	8.3 8.9	13.7 13.8	Methyl alc.-ether	2400	5,160	85

^a Maximum absorption.^b Acetyldehydrophenylalanine has a maximum at 2800 Å, $\epsilon = 18,000$.^c Acetyldehydrovaline does not exhibit a maximum abs., its $\epsilon_{2400\text{Å}} = 7700$.^d All melting points uncorrected in °C.^e In Ångstroms.

TABLE II

Molar Extinction Coefficient (ϵ) of Addition Products (in 80% Ethanol)

Dehydropeptide ^a	Added reagent	Wavelength	ϵ
		Å	
Acetyldehydroalanine	—	2400 ^a	6,050
Acetyldehydroalanine	Benzyl mercaptan	1400	1,160 ^b
Acetyldehydroalanine	Phenyl mercaptan	2400	850 ^b
Acetyldehydroalanine	Benzylamine	2500 ^a	245
Acetyldehydroalanine	Dimethylamine	2400	20
Acetyldehydroalanine	Methylamine	2400	20 ^b
Acetyldehydrophenylalanine	—	2800 ^a	18,000
Acetyldehydrophenylalanine	Benzyl mercaptan	2800	4,400
Acetyldehydrophenylalanine	Phenyl mercaptan	2600 ^a	4,420

^a Maximum absorption (9).^b No peak of absorption.

^c The molar extinction coefficient of chloroacetyldehydroalanine at the absorption maximum of 2400 Å is 6550 (9). The coefficient for the addition (and replacement) products with methylamine and with dimethylamine, namely, N-methylglycyl- α -amino- β -methylaminopropionic HCl, and N,N-dimethylglycyl- α -amino- β -dimethylaminopropionic acid HCl, is, respectively, 44 and 48 (8).

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Alkali Conjugation of the Unsaturated Fatty Acids¹

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INTRODUCTION

Moore (1) first postulated that the absorption bands developed by prolonged saponification of fatty acids were due to the shifting of the double bonds to a conjugated position. This was later proved independently by him (2) and by Kass and Burr (3), who isolated pseudo-elostearic acid from saponified linseed oil. Moore (1) and Burr (4) showed that the final intensity of the developed bands depended upon the degree of unsaturation of the fat used, and that whenever linolenic acid was present a band appeared at about 2700 Å in addition to the one at about 2350 Å.

Much earlier it had been shown by Edisbury, Morton and Lovern (5) that the rate of development of absorption bands in cod liver oil was very rapid in a high boiling solvent containing potassium hydroxide. These authors, however, attributed the bands to products of cyclization rather than conjugation. Kass, Miller, Hendrickson and Burr (6) introduced ethylene glycol as a useful solvent in which the conjugation of linoleic acid goes smoothly and rapidly to a reproducible empirical endpoint within 30 min. This procedure was used as a direct and rapid analytical method which gave values for the linoleic acid content of many vegetable fats agreeing closely with those calculated from iodine and thiocyanogen numbers (7,8). Mitchell, Kraybill and Zscheile (9)

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extended the glycol-KOH method to include linolenic acid, and Beadle and Kraybill (10) established tentative empirical constants for arachidonic acid. Since that time the method has been widely used with various modifications for fat analysis (11-18).

Unfortunately, alkali produces all possible types of conjugation. For example, in an acid with 4 double bonds, tetraene, triene, and diene conjugation are produced. The accuracy of an analysis of mixed acids is limited, therefore, by the constancy of the intensity ratios between the bands characteristic of these polyenes. These ratios are known to be affected by time, temperature, solvent, and alkali concentration. In the present paper a study has been made of the conjugation of linoleic, linolenic, and arachidonic acids with time and alkali concentration varying, temperature being constant.

EXPERIMENTAL

The ethyl linoleate, ethyl linolenate, and methyl arachidonate used in these studies were prepared from their respective polybromides and had iodine numbers of 160.6, 227.0, and 316.5, respectively. The conjugation reactions were performed in loosely stoppered 1×8 in. test tubes containing 5.0 ml. of an ethylene glycol solution of KOH whose concentration was measured titrimetrically. The test tubes containing the mixture were placed in an oil bath held at 178°C . 15 min. prior to the addition of 15-25 mg. samples of the esters in 0.5×1.0 cm. vials. The mixtures were then shaken violently and replaced in the bath for the desired length of time, when they were removed and plunged into cold water to stop the reaction. The samples and appropriate blanks were diluted with absolute methanol prior to spectrophotometric examination with a Beckman spectrophotometer.

RESULTS AND DISCUSSION

Effect of KOH Concentration on the Degree of Conjugation

To learn the effect of variation of alkali content of the medium on the conjugation reaction, a series of experiments was performed on the 3 esters in which a standard time of 30 min. heating at 178°C . was used, but in which the KOH content of the glycol solution was varied. The results of these experiments are found in Figs. 1 and 2. It will be seen that maximal diene conjugation is induced in all 3 esters by concentrations of KOH of 10 g./100 ml. or less.

Maximal yields of conjugated triene from linolenic acid are obtained at a KOH concentration of about 25 g./100 ml. at which concentration the formation of diene from this acid is greatly suppressed (Fig. 1). A

somewhat similar picture is seen with arachidonate, where maximum yield of tetraene conjugation is found at about 23 g. KOH/100 ml., and where the yields of both diene and triene conjugation are greatly reduced (Fig. 2). Spectra of the alkali-isomerized arachidonate showed a pronounced absorption maximum at 3450 Å indicating the presence of some pentaene ester in the preparation which was made from the polybromides of fresh hog liver fat. Maximum pentaene conjugation was induced by a concentration of about 20 g. KOH/100 ml.

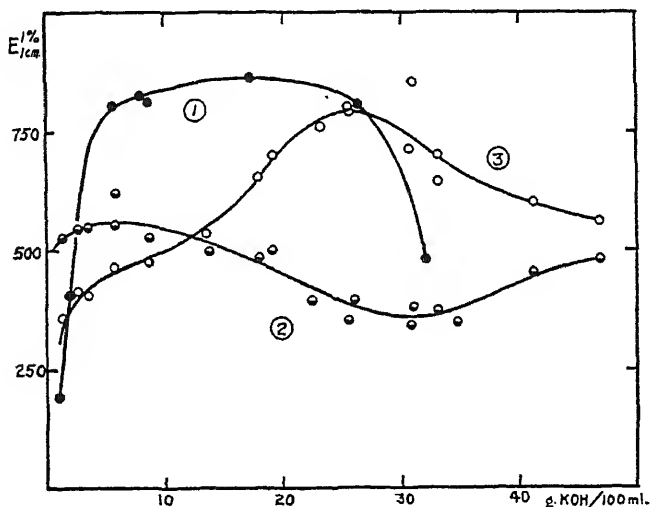


FIG. 1. Effect of KOH concentration upon linolenic and linoleic acid conjugation with 30 min. treatment at 178°C.

- (1) Diene conjugation of linoleic acid.
- (2) Diene conjugation of linolenic acid.
- (3) Triene conjugation of linolenic acid.

Effect of Time upon Conjugation

It was realized that changes in the KOH concentration might alter the rate of the reaction as well as the yield of conjugated material present at the end of the arbitrarily chosen 30 min. period. That is, the extinction values observed at the end of 30 min. may not necessarily be the maximum possible value, but may actually lie on either an ascending or descending portion of the time curve. Therefore, time curves were run for each of the acids at the approximately optimum KOH

concentration of 22–23 g./100 ml. (Figs. 3 and 4). It is apparent that the use of high KOH concentrations increases the rate of the diene conjugation. Mitchell *et al.* (9), who used about 7.3 g. KOH/100 ml., found that maximum conjugation of linoleic acid was attained in about 30 min., and that maximum diene conjugation from linolenic acid was attained in about 20 min. With 22 g. KOH/100 ml. these maxima were

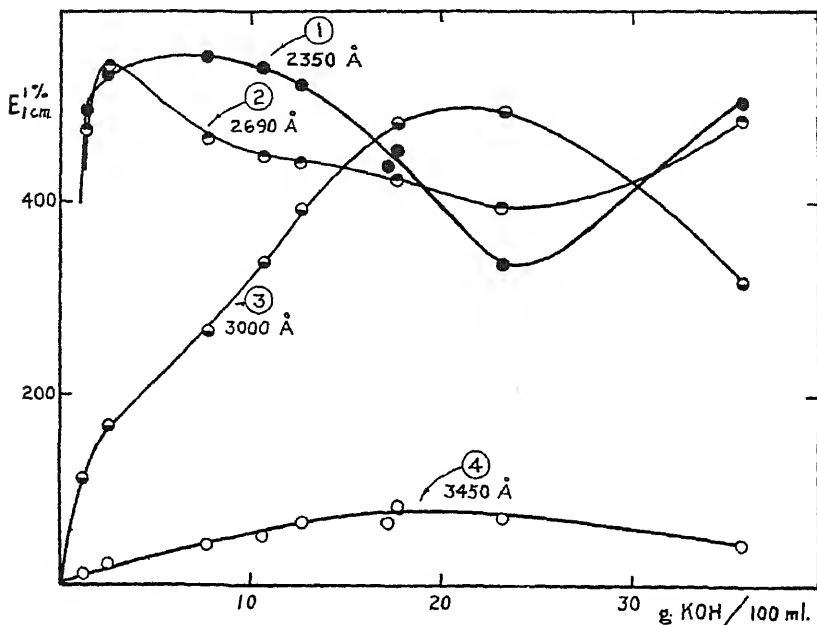


FIG. 2. Effect of KOH concentration upon arachidonic acid conjugation with 30 min. treatment at 178°C.

- (1) Diene conjugation.
- (2) Triene conjugation.
- (3) Tetraene conjugation.
- (4) Pentaene conjugation (impurity).

attained in 10 and 5 min., respectively. At this high concentration of KOH, triene conjugation of linolenic acid does not reach a maximum within 40 min., although it does at the lower concentration used by Mitchell *et al.*

The study of the effect of time upon arachidonate conjugation at 3 different KOH concentrations is shown in Fig. 4. It will be seen that, as

the KOH concentration is increased, both the maximum yield of conjugated tetraene and the rate of formation are increased. (This is likewise true of conjugated pentaene formation.) The optimum conditions for conjugated tetraene and pentaene formation at 178°C. were found to be treatment with 22–23 g. KOH/100 ml. for 8–10 min. It is apparent that excessive KOH or prolonged treatment leads to destruction of the con-

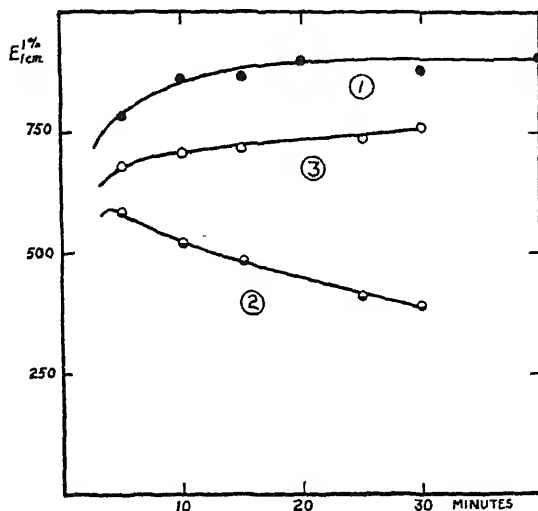


FIG. 3. Effect of time on conjugation of linoleic and linolenic acids at 178°C.

- (1) Linoleic acid conjugation using 22.3 g. KOH/100 ml.
- (2) Linolenic acid conjugation using 23.0 g. KOH/100 ml.: diene.
- (3) Same as 2: triene.

jugated systems. Use of a lower temperature might eliminate this rapid destruction, but temperature studies have not been made in the present investigation.

Destruction of pseudo-Eleostearic Acid

Brice and Swain (15) have shown that although, under the conditions which induce conjugation of isolated double bonds, conjugated trienoic acids (α -, β -, and *pseudo*-eleostearic acids) are decomposed, as evidenced by a markedly decreased absorption at 2700 Å. Conjugated dienoic acid (10, 12-linoleic) is comparatively stable. This decreased absorption could be due to destruction of the conjugated material or

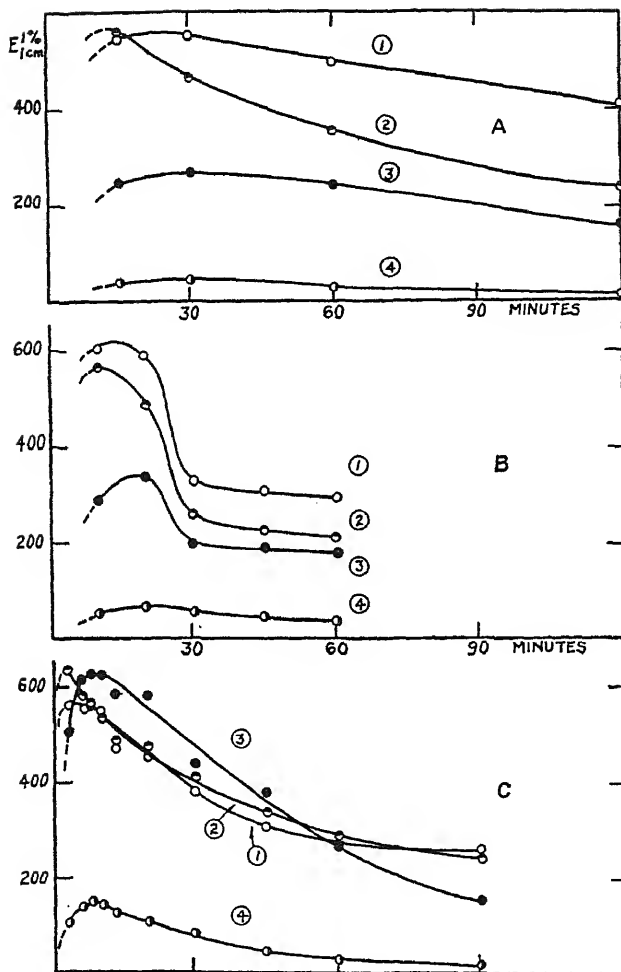


Fig. 4. Effect of time upon conjugation of arachidonic acid at 178°.

(A) 7.8 g. KOH/100 ml.

(B) 11.6 g. KOH/100 ml.

(C) 23.5 g. KOH/100 ml.

(1) Diene conjugation.

(2) Triene conjugation.

(3) Tetraene conjugation.

(4) Pentaene conjugation (impurity).

to deconjugation. If the latter occurred, the formation of diene from pure triene should be demonstrable. Such diene production would indicate the reversible nature of the conjugation reaction.

To test this possibility, small samples of *pseudo*-eleostearic acid, the solid conjugated isomer of linolenic acid, were treated with the conjugating reagent in which the concentration of KOH was 6.9 g./100 ml. This low concentration was chosen because it favors the formation of diene from linolenic acid (Fig. 1). The extinction at zero time was determined on a sample dissolved in an identical quantity of ethylene glycol-KOH without heating. The change in extinction coefficients at 2350 Å and 2680 Å with time is shown in Table I.

TABLE I
*System Ethylene Glycol-KOH-pseudo-Eleostearic Acid; 178 C.;
6.9 g. KOH/100 ml.*

Time min.	$E_{1\text{ cm. at } 2350\text{ Å.}}^{1\%}$	$E_{1\text{ cm. at } 2680\text{ Å.}}^{1\%}$
0	196	1862
8	284	1720
16	278	1650
30	250	1530
50	271	1460
72	224	1245
115	201	1066

It will be seen that early in the treatment, the extinction coefficient increases at the diene region while the triene decreases. Although the change in extinction coefficient at the 2 wave lengths indicates possible formation of diene from triene during the first few minutes, there was no evidence of a new band peak at 2350 Å proportional to the loss in triene conjugation. It is apparent that the continued disappearance of triene is not due to simple reversal of the conjugation reaction, but probably involves polymerization. Therefore, the different degrees of conjugation induced under conditions described earlier in this paper are probably not due to a shifting of a true equilibrium, but to variations in the rates of formation and destruction.

*Proposed Modifications of a Method of Analysis
Adapted to Polyethenoid Acids*

Kahnke (18), using similar conditions, has recently found that 8 min. reaction times gave optimal yields of conjugated hexaene as well as

pentaene and tetraene from cod liver oil, verifying the results reported here. The conditions suggested for maximal yields of conjugated tetraene, pentaene, and hexaene from the nonconjugated isomers occurring in animal tissue lipides are 22-23 g. KOH/100 ml. ethylene glycol solution and 8.0 min reaction time at 178°C. Using these conditions the extinction coefficient for conjugated tetraene (3000 Å) from arachidonate has been raised to $E_{1\text{ cm.}}^{1\%} = 622$, as compared with 258 under the conditions used by Beadle and Kraybill (10). The sensitivity for this acid has therefore been more than doubled.

Studying the reproducibility of the absorption bands produced from cod liver oil under these proposed conditions, Rieckehoff (19) found the $E_{1\text{ cm.}}^{1\%}$ for the various absorption bands as follows:

2325 Å 179.5; S. E. \pm 6.12; 2700 Å 171.8; S. E. \pm 3.76;
3000 Å 149.1; S. E. \pm 1.26; 3450 Å 113.9; S. E. \pm 0.029;
and 3725 Å 40.03; S. E. \pm 0.316. (S. E. = standard error)

It is thus apparent that, if one is primarily interested in the measurement of the polyunsaturated fatty acid, this method gives a more sensitive measure of their concentration in fats, and that the reproducibility is satisfactory. On the other hand, these conditions should not be used for the measurement of linoleic or linolenic acids because of the low yield of their respective conjugated isomers and the poor reproducibility of these absorption bands. It is also recommended that, in using this or other modifications of the alkaline conjugation, the investigator determine his own empirical constants with pure unsaturated acids or reference oils if at all possible.

SUMMARY

1. The effect of KOH concentration upon the alkaline conjugation of polyunsaturated fatty acids has been studied. Maximum yields of diene from linoleic acid are produced over a wide range of KOH concentrations. To produce maximum conjugated triene, tetraene, and pentaene from their respective unconjugated acids the concentration of KOH should be 22-23 g./100 ml.

2. The development of conjugation with time at the optimum alkali concentrations have been studied. From these studies it is apparent that the optimum time of treatment is 8.0 min. at 178°C. Using these conditions the sensitivity of the method for arachidonate is more than doubled.

3. From a study of the behavior of *pseudo*-eleostearic acid in alkaline glycol it is apparent that the influence of varying conditions upon the yield of conjugated material is not due to a shifting of a true equilibrium.

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Replacement of CO₂ in Heterotrophic Metabolism ¹

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INTRODUCTION

Organisms completely deprived of carbon dioxide fail to multiply (Valley and Rettger (8), Gladstone *et al.* (2), and others). The phenomenon has thus far not been adequately explained. It may be postulated that the gas enters into the synthesis of essential intermediates. If this assumption is correct, then the intermediates in question should replace carbon dioxide. Since bacteria are capable of fixing carbon dioxide on pyruvic acid to form succinic acid *via* oxaloacetate (Wood-Werkman reaction), it should be possible to replace carbon dioxide with compounds arising from the fixation. Such a replacement results in not only normal but, in many instances, enhanced growth.

Although Lwoff and Monod (4) have recently shown that combinations of both succinate and DL-glutamate will replace CO₂ and Lyman *et al.* (5) that CO₂ and vitamin B₆ are important factors in modifying the amino acid requirement for some lactic acid bacteria, no reports have thus far appeared in the literature on the extent to which CO₂ can be replaced *in vivo* by substances occurring in the proposed Krebs' cycle or their metabolic precursors.

The present study was undertaken to determine the behavior of members of the Krebs' cycle as substitutes for CO₂. It has been found that none of the C₃ compounds tested replaces CO₂, whereas the C₆ compound tested, *e.g.*, *cis*-aconitic acid, in the case of both *Escherichia coli* and *Aerobacter aerogenes*, and citric acid, only in the case of *A. aerogenes*, substitute in relatively high concentration. The C₄ and C₅ compounds apparently are used best by both organisms as substitutes for CO₂.

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METHODS

The organisms employed in these experiments were *E. coli* and *A. aerogenes*.

The basal medium consisted of 0.8% K_2HPO_4 , 0.4% $(NH_4)_2SO_4$, 10% tap water (for inorganic ions), and 0.8% glucose, made up with distilled water to the desired volume. The glucose was autoclaved separately to prevent caramelization and added to the medium aseptically. The pH was adjusted to 6.8. The compounds to be tested were normally added to the medium before autoclaving, except in the case of oxaloacetic acid. A solution of the sodium salt of this acid was sterilized by filtration through a Seitz filter and added aseptically to the medium.

The inoculum was a 24 hr. culture grown in 10 ml. of the basal medium. Transfers were made with the same loop (3 mm. in diameter) daily so as to insure uniform growth throughout the experiments. The tube from which the inoculum was made was always checked and adjusted in turbidity to give the same reading on the photoelectric colorimeter. The culture was checked periodically for purity.

CO₂-Absorption

The air was first passed through a sterile, cotton-filled tube and then freed from CO_2 by passing it through an alkali tower filled with glass beads containing 30% NaOH with phenolphthalein as an indicator. Since we have found that the rate of growth depends not only upon the addition of certain compounds but upon the rate of aeration and the porosity of the aeration discs as well, proper precautions were taken. The same sintered glass disc was used in all experiments and the rate of aeration was kept constant by introducing a manometer into the aerating system immediately after the culture flask. Five volumes of air were passed per min.

The reaction flask contained 100 ml. of basal medium, to which 1% of inoculum by volume was added aseptically, unless otherwise stated.

Oxaloacetic acid was determined by the aniline citrate method (1). All experiments were conducted at 30°C. Growth was measured by turbidimetric readings on the Klett-Summerson photoelectric colorimeter with a 660 m μ light filter.

All readings were made after incubation for 18 hr. unless otherwise stated.

RESULTS

Table I shows the effect of different atmospheric conditions on the growth of *E. coli*. Although growth is appreciably reduced by the absence of CO_2 , it is not completely inhibited, even when the inoculum is considerably diluted. That the gas is necessary for the optimal growth of bacteria is clearly seen when the results of Table II are compared with those of Table I. The results show (Table II) that irrespective of the size of the inoculum, but depending on the time, maximum growth reached in the absence of CO_2 is far less than under the same conditions with CO_2 present. When air is allowed into the culture flask after

TABLE I
Effect of Different Atmospheric Conditions on the Growth of E. coli

Time in hours	Aeration with air	Aeration with CO ₂ -free air	Aeration with CO ₂ -free O ₂	No aeration
12	225	0	0	80
10	230	30	0	80
24	245	43	2	80
42	275	57	13	85
48	300	57	13	85

Results in terms of turbidity readings. 0 indicates no growth. Total volume, 101 ml. Temp. 30°C.

maximum growth has been attained, little or no further increase in growth is observed under the same experimental conditions.

The concentration of CO₂ in the medium appears to be an important factor in determining conditions for optimal growth (Table III). The concentration of CO₂ in the air, although small, seems to be favorable. In the presence of 5% CO₂ a decrease in growth is noted and a further decrease takes place in 10% CO₂. The pH of the medium in the presence of 10% CO₂ did not change appreciably in 18 hrs., indicating that the reduction in growth was due to the CO₂ and not to a change in pH. Aeration with 100% CO₂ resulted in total inhibition of growth even though the medium was highly buffered. These results further substantiate the toxicity of CO₂ at high concentrations.

Why CO₂ is necessary for growth is not certainly known. However, since Wood and Werkman (9) have shown that CO₂ is fixed by hetero-

TABLE II
Effect of Size of Inoculum on the Growth of E. coli in the Absence of CO₂

Aeration time in hrs.	(CO ₂ -free air)	12	18	24	30	42	48	53	66	72
Dilutions	Original inoculum	0	30	43	57	57				
	1:10	0	0	8	9	50	57	57		
	1:100	0	0	0	3	10	25	65	65	65

Results in terms of turbidity readings; 0 indicates no growth. Total volume, 101 ml. Temp. 30°C.

TABLE III
Effect of CO₂ on the Growth of *E. coli*

Concentration of CO ₂ in the aeration air	Normal conc.	5%	10%	100%
Growth	230	122	55	0
Final pH of the medium	6.0	6.6	6.2	5.6

Results in terms of turbidity readings; 0 indicates no growth. Total volume 101 ml., Temp. 30°C. Incubation time, 18 hr.

trophic organisms to form oxaloacetic acid and subsequently members of the tricarboxylic acid cycle, the replacement of CO₂ by these intermediates or their potential precursors, was determined.

The C₄ dicarboxylic acids apparently substitute for CO₂ to the same extent, indicating, in conformity with the proposals of Szent-Györgyi, a similar mode of action of these compounds (Fig. 1).

Oxaloacetic acid is not immediately spontaneously decarboxylated, even at 30°C. (Table IV). The acid may be found unchanged in the reaction flask after 12 hr. The results show that, if the intact molecule of oxaloacetic acid is necessary for growth as such, it is present.

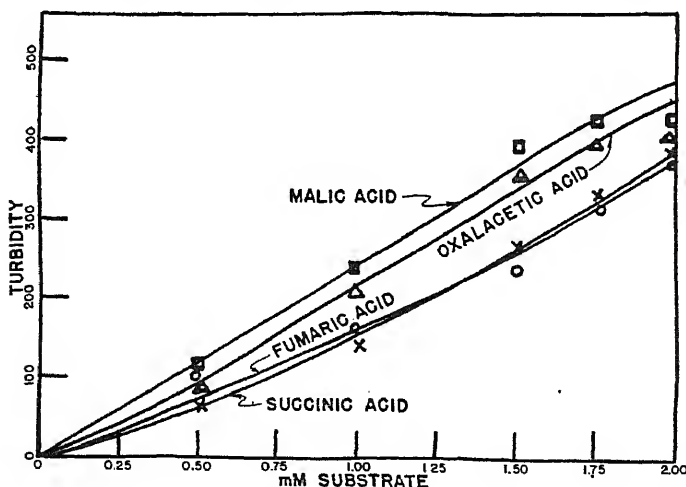


FIG. 1. Total volume 101 ml. containing 100 ml. of basal medium, 1% inoculum of *E. coli* and various concentrations of the C₄ dicarboxylic acids. Incubation time, 18 hr. Temp. 30°C. Gas phase CO₂-free air.

TABLE IV
Relationship of Disappearance of Oxaloacetic Acid to Growth

Time in hours	0	3	6	9	12
Oxaloacetic acid; μ l	350	239	124	96	29
Growth	0	0	20	40	55

Oxaloacetic acid was determined on the Warburg respirometer as CO₂. Temp., 30°C. Total volume per cup, 2.3 ml. containing 1.6 ml. test solution, 0.3 ml. citric acid, and 0.4 ml. aniline citrate.

Growth measured as turbidity. A reading of 0 indicates no growth. Incubation temp., 30°C. Continuous aeration with CO₂-free air.

Since the C₄ acids are members of the tricarboxylic acid cycle, other members of the cycle should replace CO₂ if the cycle is reversible or if side reactions are also reversible (Figs. 2 and 3).

α -Ketoglutaric acid and glutamic acid substitute to approximately the same extent, but considerably less of these compounds is necessary to give the same amount of growth as obtained with the C₄ acids. These data, therefore, apparently indicate the possibility of a fixation over and above the Wood and Werkman reaction.

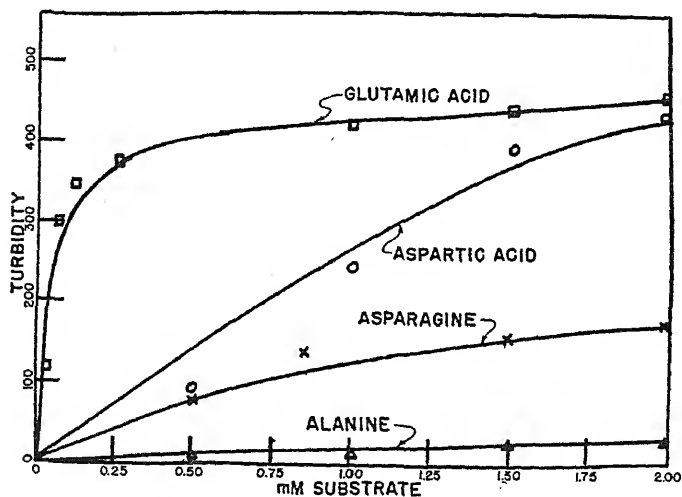


FIG. 2. Same conditions as in Fig. 1. Effect of various concentrations of amino acids on the growth of *E. coli*.

The effect of asparagine is relatively small compared with the other compounds. This result may be expected if we assume the substitutive effect of this compound to take place through aspartic acid.

An explanation of the difference in growth resulting from aspartic acid and from glutamic acid is that the conversion of glutamic acid to α -ketoglutaric acid is much more efficient than the conversion of aspartic acid to oxaloacetic acid, or that glutamic acid is of primary importance to the metabolism of the cell and cannot be synthesized adequately in the absence of CO_2 .

None of the C_3 compounds tested substituted for CO_2 , since there was no CO_2 in the medium to permit an initial fixation reaction, which apparently is necessary for optimal growth.

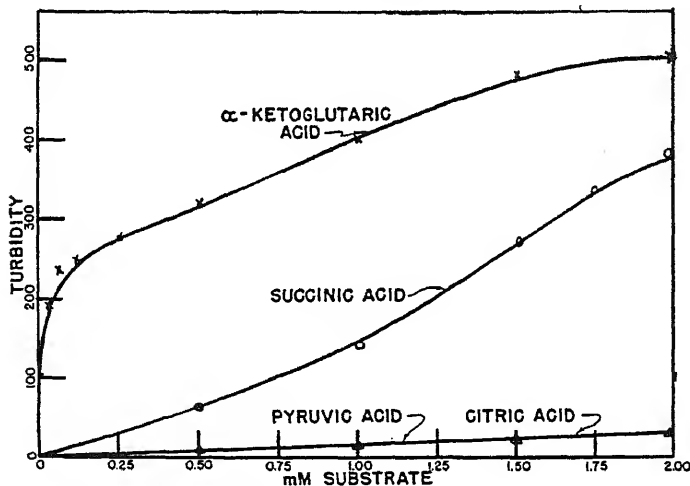


FIG. 3. Same condition as in Fig. 1. Effect of various concentrations of the C_3 , C_4 , C_5 , and C_6 compounds tested on the growth of *E. coli*.

Stetten and Schoenheimer (7) fed isotopic L (-)-proline to rats and isolated various amino acids from the carcass and organ proteins. They found deuterium as well as N^{15} in the isolated glutamic acid. Krebs (3) suggests that arginine may be converted through α -keto- δ -aminovaleric acid to α -ketoglutaric acid. These compounds were, therefore, tested as substituents for CO_2 . It is of interest that arginine and proline do, whereas histidine and lysine do not, replace CO_2 in the metabolism of *E. coli* (Table V).

TABLE V

Effect of Arginine, Proline, Histidine, and Lysine on the Growth of E. coli in the Absence of CO₂

Compound	Amount added in mM	Growth
Arginine	1	150
Proline	1	100
Histidine	1	30
Lysine	1	30

A reading of 30 indicates no growth due to the addition compound. Total volume, 101 ml. Temp., 30°C. Incubation time, 18 hr. Aerated with CO₂-free air.

Experiments have been conducted to indicate whether the effects of substituting compounds are additive (Table VI). When two such compounds are added to the culture flask, each acts as if it were present alone.

The action of the C₆ acids depended on the organism (Table VII). Whereas *cis*-aconitic acid can substitute for CO₂ to some extent with both *E. coli* and *A. aerogenes*, citric acid is effective only for the latter. The effect of both of these compounds is low as compared to the C₄ and C₅ acids.

Since Slade *et al.* (6) have shown that acetic acid can be condensed by *A. indologenes* to form succinic acid, attempts have been made to use acetic acid to substitute for CO₂. No satisfactory results have been obtained. Equimolar amounts of pyruvic acid and acetic acid also gave negative results. These results may be accounted for by the environmental growth conditions.

In all cases where growth was retarded in the absence of CO₂, abundant growth did take place upon the addition of any of these com-

TABLE VI

Additive Effects of Compounds Substituting for CO₂ in E. coli Metabolism

Compound	Succinic acid	Fumaric acid	Succinic + fumaric	α -Ketoglutaric acid	Succinic + α -ketoglutaric
Amount added; mM	1	1	1 of each	.035	1 of succinic + .035 α -ketoglutaric
Growth	120	140	305	220	320

Total volume, 101 ml., temp. 30°C., 18 hr. incubation period. Aerated with CO₂-free air.

TABLE VII
*Effect of cis-Aconitic Acid and Citric Acid on the Growth of E. coli
 and A. aerogenes in the Absence of CO₂*

Organism	Compound	mM of compound added	Growth
<i>E. coli</i>	<i>cis</i> -Aconitic acid	1	70
	Citric acid	1	30
<i>A. aerogenes</i>	<i>cis</i> -Aconitic acid	1	65
	Citric acid	1	52

A reading of 30 indicates no growth due to the addition compound. Total volume, 101 ml. Temp., 30°C. 18 hr. incubation time.

pounds which were shown to substitute for CO₂. This phenomenon was observed even when no further increase of growth resulted upon the introduction of ordinary air (Table VIII).

TABLE VIII
*Effect of Air and α-Ketoglutaric Acid on the Growth of E. coli after Maximum Growth
 Has Been Reached in the Absence of CO₂*

Maximum growth in absence of CO ₂	Addition of CO ₂	Addition of α-ketoglutarate
65	80	150

Total volume of solution 96 ml (5 ml. removed to check turbidity). Amount of α-ketoglutarate added is equivalent to 2 mg./ml. Readings were made 8 hr. after maximum growth in the absence of CO₂ took place. Temp., 30°C.

DISCUSSION

The limitation of the results thus far obtained do not permit any definite statements concerning the mechanism by which any of the compounds tested substitute for carbon dioxide. However, within the limits of our experimental data we can assuredly say that the gas functions as a metabolite and not merely as an environmental factor, *e.g.*, by affecting the pH of the medium or the permeability of the cell wall.

The evidence points to an orderly manner in which the substitution of the various compounds takes place. That this is not a general effect is indicated by the fact that compounds such as alanine, pyruvic acid, histidine, lysine or citric acid do not substitute for CO₂ with *E. coli*.

A. aerogenes, which normally utilizes citric acid as a carbon source, can use this acid instead of CO₂.

This last observation may prove to be of considerable importance. If we were to assume that our results indicate further evidence for the possible operation of the proposed Krebs' cycle in *E. coli*, it may be that citric acid occurs in that cycle in the case of *A. aerogenes* and not in *E. coli*.

It is of considerable interest that, in addition to the compounds normally occurring in the proposed Krebs' cycle, a number of amino acids can also be used by these organisms in place of CO₂. The manner in which they substitute for the gas cannot be stated with any degree of certainty. However, two main paths of action are possible:

(1) The amino acids substituting for CO₂ are converted by transaminations or similar reactions to the various dicarboxylic acids and eventually to oxaloacetic acid, which is required by the cells as a catalyst in normal aerobic metabolism. This implies a catalytic function of these compounds.

(2) The substitution of the C₅ acids, *e.g.*, glutamic and α -ketoglutaric acid, to a greater extent than any of the other compounds tested is suggestive of a further fixation of CO₂ over and above the Wood and Werkman reaction in normal metabolism, involving the formation of a C₅ compound which can be used more efficiently by the cell. It is possible, therefore, that many of the compounds supplied in the absence of CO₂, particularly the amino acids, function in more than one way. They may supply oxaloacetic acid and/or function as products which would normally arise during the metabolism of the cell.

Our experimental results warrant the conclusion that the compounds added do not function by merely supplying CO₂ to the organisms. If this were the case, the inclusion of CO₂, after maximum growth has been obtained in its absence, should give a corresponding increase. This was found not to be the case.

SUMMARY AND CONCLUSIONS

A number of compounds have been found which, in the case of *E. coli*, substitute for CO₂ and give not only normal but, in many instances, enhanced growth. These compounds include the amino acids: arginine, proline, aspartic and glutamic acids; the dicarboxylic acids: succinic,

fumaric, malic, oxaloacetic, and α -ketoglutaric acids; and one tricarboxylic acid, namely, *cis*-aconitic acid. All of these compounds are constituents of the Krebs' oxidation cycle, or their metabolic precursors.

Although the mechanism of this substitution has not been worked out as yet, the evidence thus far points to an orderly manner in which this phenomenon takes place. That this is not a general effect is indicated by the fact that compounds such as alanine, lysine, pyruvic or citric acid and histidine, cannot be used by the organism in place of CO_2 .

The results indicate that the degree of substitution of the various compounds is quantitatively significant.

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Storage of Vitamin A in Rats Fed Cryptoxanthine and Certain Other Carotenoids with Parallel Data on Absorbability¹

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INTRODUCTION

Recently it was demonstrated (1) that rats fed all-*trans*- β -carotene or its stereoisomers stored vitamin A in amounts that were proportional to the abilities of these provitamins to promote growth (2). Rats fed α -carotene, however, stored less vitamin A than that expected on the basis of the growth-promoting power of the pigment (1). In the present study another provitamin A, cryptoxanthine, was fed to depleted rats in amounts calculated to produce measurable stores of vitamin A. α -Carotene and β -carotene served as standards of comparison, and the relative absorbability of the 3 pigments was measured at various levels of intake. The effect of lutein on vitamin A storage was also determined.

MATERIALS AND METHODS

α - and β -Carotene were prepared from a commercial crystalline carotene in the manner described previously (1). Lutein was prepared as follows: 1 g. of a xanthophyll³ mixture in cottonseed oil was saponified in 50 ml. of 10% KOH in ethanol by refluxing for 30 min. The pigments were transferred to ethyl ether in several extractions, and the extracts pooled and allowed to stand over night at a temperature of -6°C . The solution was filtered, the filtrate evaporated to dryness under vacuum, and

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³ A 5% mixed xanthophyll concentrate supplied by Midwest Extraction Co., Rockford, Illinois.

the residue taken up in 30 ml. of Skelly Solve B (b.p. 64–68°) to which 5 ml. of benzene had been added. The solution was then chromatographed on a mixture of 10 parts of Hyflo Super-Cel⁴ and 1 part of $\text{Ca}(\text{OH})_2$. On development with Skelly Solve B, the main band, lutein, occurred near the top of the column. It was eluted with 5% ethanol in Skelly Solve B, and rechromatographed 3 times. The lutein was crystallized from CS_2 by the addition of ethanol (3) and was finally dried under high vacuum at a temperature of 78°C. for 5 hr. The absorption spectrum was measured in absolute ethanol in a Beckman Spectrophotometer, and the maxima found to be at 447 $\text{m}\mu$ and 474–476 $\text{m}\mu$, with the specific absorption coefficients of 286 and 263, respectively.

Cryptoxanthine was prepared from persimmons (*Diospyros kaki* L.) by a method somewhat similar to that of Schoen (4). Eight and one-half kg. of ripened fruits were broken up by hand and kept under 4–5 l. of methanol for 24 hr. They were strained through several layers of cheese cloth and the excess liquid pressed out, ground in a food grinder, and the dehydration with methanol repeated. The methanol was filtered off, and the pulp partially dried in a current of air for 2 hr. It was then extracted twice with a total of 8 l. of Skelly Solve B-methanol (3:1) and once with 3 l. of Skelly Solve B-acetone (3:1). The pooled extracts were washed thoroughly with water to remove methanol and acetone, dried over anhydrous Na_2SO_4 , and the Skelly Solve B solution concentrated to 25 ml. by evaporation under vacuum at 45–50°C. Saponification was carried out at room temperature for 24 hr. by the addition of 250 ml. of ethyl ether and 200 ml. of 10% methanolic KOH. The mixture was washed free of KOH and methanol, the ether removed under vacuum, 100 ml. of Skelly Solve B were added, and the solution was allowed to stand at –6°C. for 48 hr. A waxy material was filtered off, and the solution chromatographed on $\text{Ca}(\text{OH})_2$ -Hyflo Super-Cel (3:1) with Skelly Solve B-benzene (6:1) as the developing agent. The orange colored main zone occurring near the top of the column, was removed and rechromatographed 4 times. It was crystallized from benzene-methanol and dried at 65–70°C. for 20 hr. under a high vacuum. It melted at 168°C., uncorr., showed absorption maxima in ethanol at 484–5 and 452 with a specific absorption coefficient of 241 at the highest maximum. A mixed chromatogram with an authentic sample⁵ indicated no separation of zones.

Weanling rats were depleted of vitamin A in the manner described previously (1). Upon depletion, the rats were divided into groups of 8 or 9, comparable in weight and sex, and each group was given one of the carotenoids (Table I) daily for 15 days. The daily supplement of carotenoid and 0.5 mg. of α -tocopherol was dissolved in 3 drops of cottonseed (Wesson) oil which were dispensed from calibrated droppers (1, 5). Lutein was poorly soluble in the oil, and hence supplements containing it were dispensed in a volume of 6 drops. The animals were killed by decapitation 24 hr. after receiving the last supplement, and the livers and kidneys were analyzed for vitamin A as previously described (1, 6, 7).

RESULTS

Vitamin A Storage Due to β -Carotene, α -Carotene and Cryptoxanthine

When 20 γ of β -carotene were fed daily for 15 days to depleted rats, a total of 41.8 γ of vitamin A was found in the livers and kidneys, while

⁴ Hyflo Super-Cel obtained from Johns-Manville and Company, Chicago, Illinois.

⁵ We are indebted to Professor L. Zechmeister for furnishing us with this material.

a dosage of 40 γ daily resulted in an average total storage of 68 γ of the vitamin (Table I). Much lower stores resulted when α -carotene was fed; rats fed 40 γ of α -carotene daily contained only 20 γ of vitamin A; those fed 80 γ daily stored 33.6 γ of the vitamin (Table I). From these data it can be calculated (1) that the average ability of α -carotene to promote stores of vitamin A is 25% that of β -carotene, in agreement with previous results. Cryptoxanthine, on the other hand, which is essentially equal to α -carotene in its ability to promote growth (2, 8), proved to be at least as active as β -carotene in producing stores of vitamin A: rats fed 40 γ daily of cryptoxanthine stored 73 γ of vitamin A, as compared with

TABLE I
Storage of Vitamin A in Rats Fed Various A-Active Compounds

Supplement ^b	Wt. gain	Kidney vitamin A	Liver vitamin A	Total vitamin A ^a
	g.	γ	γ	γ
20 γ β -carotene	51	17.3	24.9	42.1 \pm 4.7
40 γ β -carotene	61	16.0	52.1	68.1 \pm 4.9
40 γ carotene + 100 γ lutein	43.5	5.6	56.1	62.7 \pm 5.3
40 γ carotene + 200 γ lutein	38	11.8	40.3	52.1 \pm 2.0
40 γ α -carotene	45	10.4	10.3	20.6 \pm 2.5
80 γ α -carotene	46	11.1	24.4	35.5 \pm 3.2
40 γ cryptoxanthine	45.5	12.1	61.5	73.6 \pm 3.8
80 γ cryptoxanthine	47.5	8.2	117.2	125.5 \pm 12.3

^a In the expression 42.1 \pm 4.7, the 4.7 is the standard error of the mean, s.e. = $\sqrt{\frac{\sum(X - \bar{x})^2}{n(n-1)}}$, where X = individual values, \bar{x} = the group mean, and n = the number of individuals.

Groups fed β -carotene or cryptoxanthine contained 9 rats/group; all others contained 8 rats/group.

^b Supplements were fed daily for 15 days.

68 γ in rats fed β -carotene. The activity of cryptoxanthine at a higher level of intake was correspondingly high: 80 γ daily resulted in body stores of 125 γ of vitamin A (Table I). Thus, since cryptoxanthine is only 57% as active as β -carotene in promoting growth (9), it follows that on the basis of international units of provitamin A administered, cryptoxanthine is twice as active as β -carotene in promoting storage, and 4 times as active as α -carotene.

Since an unequal absorption of the 3 pigments was suspected (see below) at the high levels of intake necessary for the production of stores

of vitamin A, a series of 5 depleted rats was fed 5 I.U. daily of a concentrate from halibut liver oil (enough to satisfy the needs of the animal but not enough to produce stores of vitamin A) and in addition α - and β -carotene or cryptoxanthine were fed to specific groups at levels of 5 or 10 γ daily for 14 days. Storage on the vitamin alone, or on the vitamin plus α -carotene was virtually zero. A galvanometer deflection resulted when SbCl_3 was added to liver concentrates from these groups but the color formed was a brownish yellow often seen in preparations from livers in which vitamin A storage is just beginning.⁶ However,

TABLE II
Storage of Vitamin A in Rats Fed Vitamin A and Small Amounts of Carotenoids

Supplement ^a	Daily dose	No. of rats	Weight gain	Vitamin A stores ^b
β -Carotene	γ		<i>g.</i>	
	5	6	54	10.1 \pm .8
	10	6	48	17.8 \pm 1.9
α -Carotene	5	6	41	2
	10	5	49	3
Cryptoxanthine	5	5	43	7.6 \pm 1.0
	10	5	52	19.1 \pm .8

^a Each rat received, in addition to the supplements listed, 5 I.U. of vitamin A daily. See text.

^b Figures represent total vitamin A found in livers and kidneys, except in the case of α -carotene, where kidneys only were used for the analysis. See footnote 5 of the text for explanation.

similar preparations from the rats fed the low amounts of β -carotene or of cryptoxanthine yielded clear blue colors with SbCl_3 corresponding to 10.8 and 7.6 γ on the two pigments respectively when 5 γ daily were fed, and 17.8 and 19.1 respectively on 10 γ per day (Table II). Thus, even at these relatively low levels of intake vitamin A storage was essentially equal whether cryptoxanthine or β -carotene was fed, whereas storage due to α -carotene was too low to be measured quantitatively.

⁶ When the amounts of vitamin A in the diet are borderline for producing stores, the color of liver extracts with SbCl_3 is often brown. Slightly higher amounts of vitamin A or carotene result in the development of a greenish color and still higher levels produce the blue color characteristic of the vitamin A- SbCl_3 reaction. The values reported in this paper are based only upon the latter response.

*Fecal Excretion of α -Carotene, β -Carotene and Cryptoxanthine
at Different Levels of Administration*

Healthy young rats weighing 100–125 g. were placed on the diet low in vitamin A (1) and after 7–10 days they were housed individually in wire bottom cages, and given a single dose of 2–78 γ of one of the 3 carotenoids. The desired dose was dissolved in 3 drops of cottonseed (Wesson) oil to which 0.5 mg. of α -tocopherol had been added. The feces were collected from each rat 3 times during an 84 hr. period after dosage, and the amount of carotenoid in the entire collection was determined. Since the feces from rats on the low A diet contained an ether-soluble yellow pigment equivalent to 4.0 γ of carotene per 24 hr. sample the appropriate correction was applied in all determinations. At least 3 comparable rats were used for each level of each carotenoid adminis-

TABLE III
Fecal Excretion by Rats Fed Single Doses of Various Carotenoids

Carotenoid	No. of animals	Amount fed	Amount excreted	Ingested carotenoid excreted	Average percentage excreted
		γ	γ	per cent	per cent
Cryptoxanthine	9	2.0	0.65 ^a	32	36
	3	8.7	3.8	44	
	3	17.5	6.3	36	
	3	35.0	11.9	34	
	6	70	24.8	35	
β -Carotene	9	2.0	0.70	35	40
	3	5.25	2.1	40	
	6	10.5	3.5	33	
	6	21.0	10.5	50	
	6	42	16.8	40	
	6	56	24.4	44	
α -Carotene	9	2.0	0.98	49	54
	3	7.35	4.0	58	
	3	14.7	7.5	51	
	3	29.5	17.2	58	
	3	59	27.4	46	
	3	78	45.8	59	

^a Feces from 3 animals were pooled for each analysis.

tered; when only 2 γ of pigment were fed, 9 rats were given each pigment, and the feces from groups of 3 rats pooled prior to analysis.

Each carotenoid was found to be excreted at a percentage that was characteristic for the pigment and relatively independent of dosage over the range 2–78 γ (Table III). Of the 3 pigments cryptoxanthine was absorbed best at all levels of intake, the percentage of pigment in the feces ranging from 32 to 44% of that ingested; β -carotene was intermediate, with 33–50% of the ingested pigment in the feces; whereas the excretion of α -carotene accounted for 46–59% of that ingested. While there were some irregularities in the percentages of each pigment excreted, no consistent trends were noted that associated excretion with the amount of carotenoid ingested (Table III) although the percentage of each pigment excreted was slightly lower when only 2 γ was ingested than the average for all levels, *viz.*, 32% *vs.* 36% for cryptoxanthine, 35% *vs.* 40% for β -carotene, and 49% *vs.* 54% for α -carotene.

No particular significance was attached to these differences, however, since the determination of the smallest amounts of carotenoid was less accurate than the others because of the relatively great effect that the correction due to other pigments had on the final result.

The Effect of Lutein on the Activity of β -Carotene

Rats fed relatively high levels of lutein concurrently with β -carotene stored less vitamin A than rats fed only β -carotene. The effect was slight when 100 γ of lutein were fed daily together with 40 γ of β -carotene; 62.7 γ of vitamin A were stored as compared to 68 γ when β -carotene alone was fed. However, where the level of lutein was increased to 200 γ daily, the amount of vitamin A stored decreased to 52.1 γ (Table I). Lutein also appeared to retard the growth rates of the animals: those receiving 40 γ of β -carotene daily gained 61 g. in 15 days, whereas gains of only 43 and 38 g. were observed when 100 and 200 γ of lutein, respectively, were fed with the β -carotene. Lutein did not appear to alter the absorbability of carotene. Feces were collected on the tenth day of administration, and the carotene separated chromatographically. The rats receiving β -carotene alone excreted 42% of the daily dose, while those receiving an additional 100 or 200 γ of lutein excreted 43% and 45% of the daily dose of carotene, respectively.

In the present experiment all rats received adequate amounts of vitamin E, and the results obtained agree with the conclusions of

Kemmerer, Fraps and DeMattier (10) and Kelly and Day (11) that lutein may interfere with the utilization of carotene. In the absence of vitamin E, however, lutein may actually enhance the biological value of carotene (12) presumably by retarding its oxidation to a slight extent.

DISCUSSION

The present results indicate that there is a rough similarity between the absorbabilities of α -carotene, β -carotene and cryptoxanthine and the relative abilities of these carotenoids to promote the storage of vitamin A when amounts equivalent for growth are fed to the rats. Both in completeness of absorption and in storage efficiency per international unit the order is cryptoxanthine > β -carotene > α -carotene. In growth experiments the response of one international unit is achieved by:

- 0.6 γ of ingested β -carotene (by definition),
- 1.13 γ of ingested α -carotene (8), and
- 1.07 γ of ingested cryptoxanthine (9).

The amounts of the pigments fed in the assays cited ranged from 0.6 to 4.8 γ per day, a daily intake of 2 γ of carotenoid being fairly typical of a growth experiment. At this level of intake, 35% of the ingested β -carotene was lost in the feces, 49% of the ingested α -carotene and 32% of the ingested cryptoxanthine (Table III). Thus the amounts of carotenoid absorbed for a growth response equivalent to 2.0 I.U. become:

$$\begin{aligned}0.6 \times 2 \times 0.65 &= 0.78 \gamma \text{ of } \beta\text{-carotene.} \\1.13 \times 2 \times 0.51 &= 1.16 \gamma \text{ of } \alpha\text{-carotene.} \\1.07 \times 2 \times 0.68 &= 1.45 \gamma \text{ of cryptoxanthine.}\end{aligned}$$

In other words, within the body α -carotene promotes growth better than cryptoxanthine, in spite of its inferiority in promoting the storage of vitamin A (Tables I and II).

When the fecal losses of pigment (Table III) are considered, the amounts absorbed (or destroyed) daily at high levels of intake become

$$\begin{aligned}40 \gamma \times 0.60 &= 24.0 \gamma \text{ of } \beta\text{-carotene,} \\40 \gamma \times 0.64 &= 25.6 \gamma \text{ of cryptoxanthine,} \\80 \gamma \times 0.41 &= 32.8 \gamma \text{ of } \alpha\text{-carotene,}\end{aligned}$$

with the following amounts of vitamin A stored after 2 weeks: 68 γ on β -carotene, 73 γ on cryptoxanthine, and 36 γ on α -carotene (Table I). Thus, on the basis of the amount of pigment absorbed and/or destroyed in the body, cryptoxanthine equaled β -carotene in producing stores of vitamin A, although it was only half as effective as β -carotene in promoting growth. The superiority of cryptoxanthine in the production of vitamin A cannot, therefore, be ascribed solely to its high absorbability. Furthermore, the inferiority of α -carotene in promoting vitamin A storage was evident even after a correction had been applied for the amounts of α -carotene recovered in the feces. Thus, the conclusion appears inescapable that the different carotenoids are utilized to different degrees after absorption.

With (13) has raised the question of whether carotenoids might not possess vitamin activity *per se* without first being converted into vitamin A. His question was based on the fact that chickens fed yellow corn grew better than calculations of the cryptoxanthine content of corn had led him to expect. Our present experiments do not suggest specific vitamin A activity for cryptoxanthine in the rat. Indeed, if such a property as contrasted to provitamin A activity exists at all, it should be sought in α -carotene, since, in relation to the other carotenoids, its growth-promoting power exceeds its capacity to form vitamin A.

One explanation for the low storage-promoting power of α -carotene may be a specific inhibitory influence of the α -ionone ring, a possibility suggested by the fact that lutein minimizes vitamin A storage due to β -carotene. The hydroxyl groups in the lutein do not seem to be important in this connection, since cryptoxanthine, which also contains an hydroxyl group, is a very effective source of vitamin A in the body.

Of the four carotenoids studied to date in addition to β -carotene only two, neo- β -carotene B and neo- β -carotene U (1), promoted the storage of vitamin A in amounts that were proportional to their growth-promoting capacities, whereas cryptoxanthine and α -carotene had widely different activities depending upon the biological endpoint selected. This might lend support to the idea (14) that the biological activities of the stereoisomers depend upon the extent to which they are converted to all-*trans*- β -carotene prior to absorption. According to this view, a parallelism between growth and storage should be observed in a comparative study of these isomers, since the feeding of the different isomers would essentially be a matter of feeding different amounts of the same active material, all-*trans*- β -carotene. When substances are fed,

such as α -carotene or cryptoxanthine, which do not give rise to β -carotene in the digestive tract, no parallelism results between growth and the storage of vitamin A.

SUMMARY

1. Rats depleted of vitamin A were fed either α -carotene, β -carotene or cryptoxanthine in doses of 20–80 γ /day for 15 days. Others were fed 40 γ of β -carotene together with 100–200 γ of lutein daily for a similar period. The vitamin A stores resulting in the livers and kidneys were determined colorimetrically.

2. The amounts of vitamin A found in the tissues of the rats fed α -carotene were only half those expected on the basis of its growth-promoting power. Cryptoxanthine, on the other hand, was equal to β -carotene in producing stores of vitamin A; its relative activity for storage was, therefore, twice its activity for growth. Rats fed lutein with β -carotene stored less vitamin A than those fed β -carotene alone.

3. When the various carotenoids were fed in single doses of 2–78 γ , an average of 54% of the α -carotene fed was recovered in the feces; the average excretion of β -carotene was 40%, and of cryptoxanthine, 36%. The percentage excretion did not vary with dose, nor did lutein modify the absorbability of β -carotene.

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Studies on Glycolysis of Brain Preparations. V. ¹ Affinity of Hexokinase for Glucose and Fructose

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It has been shown in the preceding papers of this series (1-4) that a high and steady rate of glycolysis is obtained in homogenates and extracts of brain if all enzymes of the glycolytic cycle are kept in step. This is especially true for the first enzyme, which induces phosphorylation of the sugar, the hexokinase, and the last enzyme, which is responsible for the dephosphorylation of the phosphorylated intermediaries, the ATPase² or apyrase. With proper concentrations of ATP and enzyme the relative affinity of the various sugars for hexokinase, as it becomes manifest in the glycolytic activity of the tissue slices, can be demonstrated in the centrifuged extracts of brain, free of cell structures and particles.

To bring this series to completion we describe further experiments to corroborate these findings. Our methods were the same as those in the preceding papers. Thus far we have had only indirect evidence that the difference of glycolytic rates in the presence of glucose and fructose with very low ATP concentration is really due to a different affinity for hexokinase, because with only a few γ 7 min. P/cc. the change of labile P cannot be accurately determined. We have found that, by lowering the sugar concentration from 0.25% to 0.05 and 0.025%, this different affinity of glucose and fructose for hexokinase

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² Abbreviations used: ATP = adenosinetriphosphate; ADP = adenosinediphosphate; ATPase = enzyme splitting first labile P-group of ATP; apyrase = adenylypyrophosphatase, enzyme splitting both labile P-groups; pyro-P = labile P of ATP and ADP; HDP = hexosediphosphate; HMP = hexosemonophosphate; DPN = diphosphopyridinenucleotide (cozymase).

can be directly demonstrated. It is especially conspicuous when the decrease of free sugar is measured by the method of Nelson (5) where the phosphate esters are removed by $\text{Ba}(\text{OH})_2$.

It was recently shown (6) that glucose is at first mainly phosphorylated only to glucose-6-phosphate in the absence of the oxidation-reduction system. However, fructose is, from the beginning, phosphorylated in great part to HDP. The difference between the disappearance of glucose and fructose is, therefore, in general, still greater than the difference for the decrease in 7 min. P.

TABLE I

Hexokinase in Brain Extract

0.5 cc. extract (1:5.5) $8 \times 10^{-2}M$ NaF. ATP with $4.5 \mu M$ pyro P = 140 γP .
All data in μM .

No.	Vol.	Time	Added sugar		Inorg. P increase	P.-transfer 7'P decrease	Sugar decrease
469	cc. 1.1	min. 12	—		1.59	0.05	
		12	gluc.	22.2	0.75	1.85	
		12	fruc.	22.2	1.02	1.68	
		12	gluc.	2.8	0.72	1.82	1.2
		12	fruc.	2.8	0.99	1.38	0.79
470	1.1	10	—		0.86	0	
		10	gluc.	2.8	0.57	1.34	1.04
		10	fruc.	2.8	0.79	0.68	0.33
489	1.3	12	—		1.44	0.20	
		12	gluc.	22.2	0.58	1.62	
		12	fruc.	22.2	0.58	1.88	
		12	gluc.	1.7	0.58	1.78	1.30
		12	fruc.	1.7	1.10	0.85	0.66

From these results we conclude that the difference between the two sugars is dependent on the concentration of the sugars as well as on the concentration of ATP. This fact is also in harmony with the turnover rates in brain slices, except that the range of sugar concentrations where the different rate becomes visible is lower in the extract than in the living tissue.

Three experiments of this type are given in Table I. Experiments 469 and 489 show that the transphosphorylation (measured as decrease of

7 min. P) is about equal for glucose and fructose in the presence of large amounts of sugar (around 0.3% or 20 μM). However, in the range of low concentrations of sugar (0.045 and 0.023% or 1.5–3 μM) the transphosphorylation remains unchanged for glucose but decreases very much for fructose. The simultaneous determination of the free sugars gives, in general, an even greater difference, so that during the time of experiment only about half as much fructose is esterified as

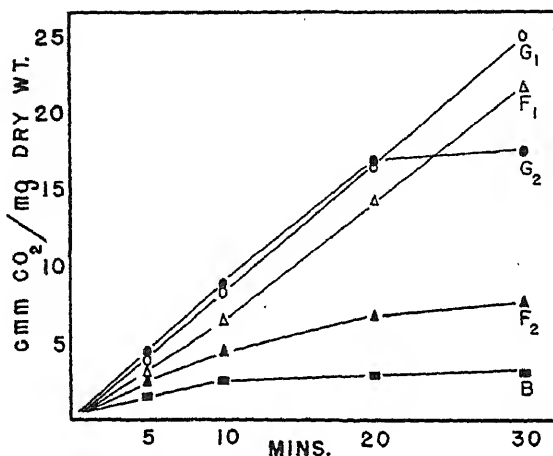


FIG. 1. Glycolysis in centrifuged extract. 0.3 cc. extract, 1:4.6 in 1.5 cc. total volume containing the sugar as well as a trace of HDP (17 γ P) as primer. (For G₂ and F₂ 0.3 cc. and 0.2 cc. extract gave nearly identical points.) 0.1 cc. cozymase (0.3 mg. DPN), 0.1 cc. ATP (35 γ pyro-P), 0.05 cc. isotonic sodium bicarbonate is tipped in from the side arm at the start. B = Blank. G₁ and F₁ = 3 mg. glucose and 3 mg. fructose, respectively. G₂ and F₂ = 0.6 mg. glucose and 0.6 mg. fructose, respectively.

glucose. Another indication for this difference is the increased dephosphorylation of ATP by the ATPase in the presence of fructose compared with glucose. This is measured by the increase of inorganic phosphate which is as much larger as transphosphorylation is smaller. In the last column of the table the disappearance of free hexose is measured by the method of Nelson (5). On the assumption that only HMP forms, the last columns should give identical figures. Because HDP also forms, the actual disappearance of hexose is smaller. This depends also on the duration of the experiments.

In Fig. 1 the same difference of metabolism of the usual concentration of sugar (0.2%) and of a low concentration (0.04%) is shown for

the glycolysis itself. The content of ATP in each sample (35 γ pyro-P/1.5 cc.) is identical with that formerly used in our studies of glycolysis of brain. Generally, it gives an equal turnover rate for 0.2% glucose and fructose (see (1)). However, with a high dilution of brain extract the turnover of fructose is sometimes a little lower, as is the case in the present experiment (No. 489). But with the low concentration of sugar the difference is exceedingly great: the rate of glucose turnover remains practically unchanged until the added sugar is exhausted which would give *in maximo* 17 mm.³ CO₂/mg. dry weight over the blank. The rate of fructose turnover, on the other hand, is less than $\frac{1}{3}$ that of glucose for the first 20 min., if the blank value is taken into account.

TABLE II
Inhibition of Hexokinase by Naphthoquinone Sulfonate

No.	Time	Sugar	mg.	10 ⁻⁴ M Naphthoqu. sulfonate	Decrease 7' P	Decrease hexose	Inhibition
	<i>min.</i>				γ	γ	<i>per cent</i>
489a	12	gluc.	4.0	8	50.0		
	12	gluc.	4.0	8	12.5		75
	12	fruct.	4.0		57.3		
	12	fruct.	4.0	8	4.2		93
489b	12	gluc.	0.3			235	
	12	gluc.	0.3	8		31	85
	12	fruct.	0.3			119	
	12	fruct.	0.3	8		0	100
485	15	gluc.	4.0		60.2		
	15	gluc.	4.0	2	43.8		27
	15	fruct.	4.0		60.8		
	15	fruct.	4.0	2	32.3		47

Even with the use of such sugar concentrations that the phosphorylation of both sugars is equal, a difference becomes apparent in the presence of some inhibitors of hexokinase. In Table II three experiments with potassium naphthoquinonedisulfonate are reproduced. The inhibition is always greater with fructose, although in the higher range of sugar concentrations the transphosphorylation is equal in the absence of the inhibitor.

The same can also be demonstrated in the inhibition of glycolysis by higher members of the narcotic series. As is shown in Table III, caprylic alcohol inhibits the turnover of fructose more than that of glucose. Here also the hexokinase is responsible for this difference. Moreover, it was recently shown (4) that HDP turnover, which does not require hexokinase, is not inhibited at all by caprylic alcohol.

TABLE III

Inhibition of Glycolysis of Glucose and Fructose by Saturated Caprylic Alcohol
0.3 cc. brain extract; 35 γ pyro-P in 1.25 cc. vol. 1 hr.

No.	Sugar	mg.	Caprylic alcohol	CO ₂	Inhibition
				mm. ³	per cent
395	—		—	24	
	gluc.	4.0	—	383	
	gluc.	4.0	+	270	32
	fruct.	4.0	—	395	
	fruct.	4.0	+	162	56
396	—		—	26	
	gluc.	4.0	—	543	
	gluc.	4.0	+	328	40
	fruct.	4.0	—	542	
	fruct.	4.0	+	228	58

We will deal here only briefly with some other inhibitors of glycolysis whose locus of attack is not the hexokinase. The enzyme ATPase in brain is not inhibited by indifferent narcotics. On the contrary, there is a transitory increase of activity in the centrifuged extracts (7), while the ATPase adsorbed on the particles is unaffected (4). Sodium azide, on the other hand, inhibits the ATPase of mammalian tissues which we have investigated thus far. This contrasts with the insensitivity of ordinary phosphatases, which are not inhibited by 1×10^{-2} *M* sodium azide. The azide inhibition of ATPase in brain is relatively weak, being about 30% in this range of concentration.

The oxidizing enzyme which catalyzes the oxidation of glyceraldehyde phosphate in the presence of phosphate and cozymase is unaffected by azide. On the other hand, it is very sensitive to sulfhydryl inhibitors. A strong inhibitor of this kind is oxidized glutathione. While a freshly prepared glutathione solution is indifferent, older solutions inhibit more and more with advancing age because of their increased content of oxidized glutathione. With 5×10^{-3} *M* oxidized glutathione, glycolysis in brain extract is progressively inhibited with an average of 66–80% in 1 hr.

DISCUSSION

The different rates of turnover of glucose and fructose in brain extracts with very low concentrations either of ATP or sugar must surely be ascribed to a lowering of the hexokinase affinity of fructose but not of glucose, while, on the other hand, in a higher range of ATP and sugar concentrations, the affinities of both sugars are equal. These experiments in themselves are no direct argument for the existence of 2 different hexokinases, glucokinase and fructokinase, according to G. T. Cori and Slein (8). However, other indirect arguments speak in favor of the idea that not only muscle and liver, as these authors have shown, but also brain and tumor tissue, and possibly all mammalian tissues, contain these 2 enzymes. The first argument is that hexokinase of yeast, which consists of only one component, shows no difference in affinity for glucose and fructose at low ATP or sugar concentrations, as we have convinced ourselves. The different extent of inhibition of turnover of glucose and fructose, as described in the foregoing, might be ascribed to a change of affinity similar to that brought about by a change in the concentrations of the reactants. However, a simpler explanation would be the presence of 2 enzymes with different sensitivity.

In tumor tissue we found still another argument for the separate existence of glucokinase and fructokinase, because in some types of tumor the relative rates of glycolysis and phosphorylation of both sugars are different from those in other types of tumors, under exactly the same experimental conditions. This can only be explained by different relative amounts of the 2 enzymes in various types of tumors. However, the dependence on concentrations of the reactants and the relative rates of turnover in slices of tumor tissue are exactly the same in all types (7).

SUMMARY

The difference of the turnover rates of glucose and fructose in centrifuged extracts of brain has thus far only been observed in a very low range of ATP concentration (about 5 γ pyro-P/cc.) in the presence of 0.2% sugar and more. In the present paper it is shown that a similar difference appears with higher ATP content in a very low range of sugar concentrations (0.05–0.025%). In this case the diminished rate of transphosphorylation between ATP and fructose can be directly dem-

onstrated in the absence of the oxidation-reduction system. Under these conditions fructose acts with about half the speed of glucose.

A difference in the same direction can also be brought about in the normal range of sugar concentration (0.2%) by inhibitors of hexokinase, naphthoquinone sulfonic acid and certain narcotics.

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Fixation of Atmospheric CO₂ in the Dark
by Leaves of *Bryophyllum*

It has recently been shown (1) that formation of acids in the dark by excised leaves of succulents such as *Bryophyllum* is intimately dependent on the partial pressure of CO₂ in the atmosphere surrounding the leaf. An evident mechanism for this effect is the possibility that leaves of succulents may in some way be capable of fixing CO₂ with the formation of carboxylic acids. That this is in fact the case has now been shown with the aid of C¹⁴O₂.

Leaves of *Bryophyllum crenatum* were excised from the plant and exposed in the dark to an atmosphere of air enriched to 5% CO₂. The entire system was then incubated for 60 hr. at 2-3°C., a time and temperature previously shown (1) to be suitable for abundant accumulation of acid by *Bryophyllum* leaves. The leaves were then dried at 70°C., ground, and fractionated.

Quantitatively reproducible uptake of C¹⁴O₂ was obtained in a series of experiments, and representative data of a typical experiment are given in Table I. Of the original radioactive CO₂ supplied to the leaves in this experiment, 23% was removed and fixed in the tissue. This corresponds to an overall fixation of 16 mg. of the 70 mg. originally supplied. The net fixation is not this large, however, and amounted to approximately 8.6 mg. of CO₂, indicating that CO₂ was simultaneously lost by respiration. Dark fixation of CO₂ takes place, therefore, on a relatively large scale in *Bryophyllum*. The tissue was acidified, extracted with ether, and the mixed acids precipitated from 85% ethanol as the barium salts. The washed mixed-acid fraction, containing mainly isocitric, citric, and malic acids (1), contained over half the fixed carbon while the specific activity of this fraction was approximately 5 times as high as that of the tissue as a whole.

The residue from extraction of the acids was next extracted with 80% ethanol and this fraction, consisting principally of soluble sugars and

amino acids, contained a further 28% of the fixed carbon. The insoluble residue of cell wall materials, protein, *etc.*, contained an appreciable amount of the fixed carbon although at a relatively low specific activity.

The data of Table I show that leaves of a typical succulent do take up CO₂ from the external atmosphere under conditions favorable to plant acid formation. The fixed carbon appears primarily in the plant acid fraction, although lesser amounts are found in other plant constituents. This fixation of CO₂ in the dark differs quantitatively, at least, from that described by Smith and Cowie (3) with leaves of sunflower, in which the bulk of the dark CO₂ uptake consists of the formation of insoluble carbonates and reaction with buffer systems. Dark fixation

TABLE I

Uptake and Redistribution of C¹⁴O₂ by Excised Leaves of Bryophyllum Crenatum
Leaves cultured at 2-3°C. for 60 hr. in 715 ml. of air enriched to 5% in CO₂

No.	Fraction	Dry wt. of fraction	Specif. act.	Total activity	CO ₂ fixed
		mg.	ct./min./mg.	ct./min./frac.	per cent
1	Initial CO ₂	70	172	12,000	—
2	Final CO ₂ ^a	61.4	151	9,264	—
3	Final dry leaf tissue	298	9.2	2,736	100
4	Organic acids (mixed)	31.5	43.1	1,361	50
5	Soluble in 80% ethanol (sugars, amino acids, <i>etc.</i>)	57.2	13.2	758	28
6	Tissue residue from 4 and 5 (cellulose, protein, <i>etc.</i>)	209	2.7	555	20

^a Determined on separate parallel sample.

of CO₂ by *Chlorella* and by barley leaves has been studied by Calvin and Benson (3) who have, however, concentrated on the fixation induced by a previous exposure to light, and which is essentially complete in less than 2 min. Formation of acids under the influence of CO₂ proceeds in *Bryophyllum* in the dark for many hours. In both cases, however, organic plant acids are formed with up to 52% of the dark-fixed carbon being recovered as carboxylic acids by Calvin and Benson. The large scale dark-fixation of CO₂ by *Bryophyllum* leaves, would appear to constitute a quantitatively important mechanism in the metabolism of this succulent.

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ERRATA

In Vol. 18, No. 3, two errata appear. On p. 502, next to the last line of text, the statement ". . . to utilize melibiose shows, however, that all β -galactosides are . . ." should read ". . . to utilize melibiose shows, further, that this α -galactoside is not utilizable." Also on p. 502, in Table IV the fermentability of neolactose indicated in Col. 3 as "-" should be changed to "++."

In the paper by C. Neuberg, H. Lustig and R. Dresel, Vol. 19, p. 165, line 5, read "reducible" for "reduced."

Book Reviews

Vitamins and Hormones, Vol. V. Edited by ROBERT S. HARRIS AND KENNETH V. THIMANN. Academic Press, Inc., New York, 1947. viii + 478 pp. with Index Vols. I to V. Price \$7.50.

The fifth volume of "Vitamins and Hormones" contains 11 excellent papers on subjects of up-to-date interest, covering a larger number of pages than the previous volumes. The welcome given this publication is a proof of its usefulness. In the present volume the first 6 papers deal with vitamins and the remaining 5 are on hormones.

N. A. Milas describes the synthesis of Vitamin A, its esters and derived compounds in a masterly way. D. Melnick and B. L. Oser discuss the limitations of the physical, chemical, and biological methods for the determination of the available vitamin contents of foods, proposing that the assay should be made on human subjects whenever possible. In a most interesting report of his personal experiments, A. von Muralt arrives at the conclusion that thiamine is essential for the normal metabolism of nerve, and also that thiamine and cocarboxylase play a part in many important reactions of nerve metabolism. The recent work on the physiological action of the pteroylglutamates in man, as well as their therapeutic properties, is competently summarized by W. J. Darby. Finally, the vitamin requirements of the chick are discussed by H. R. Bird, and those of the mouse by Harold P. Morris.

Turning to the hormones, we find Choh Hao Li and H. M. Evans, with their special authority as the original workers on the subject, review for the first time their chemical and physiological research on the properties of the growth and adrenocorticotrophic hormones. A very good and complete summary of the effect of exogenous estrogenous estrogens on the male mammal is provided by C. W. Emmens and A. S. Parkes. There is a brief account of the biology of antithyroid agents by H. A. Charipper and Albert S. Gordon, while the use of androgens in women is surveyed authoritatively by Anne C. Carter, E. J. Cohen and E. Shorr. C. S. Heller and W. O. Maddock describe the symptoms and diagnostic criteria of hypogonadism in man, discussing critically the therapeutic applications of testosterone.

The great value of these papers is that they have been written by those whose wide personal knowledge of the subjects discussed gives them the right to opine with authority and they are, therefore, very useful for endocrinologists, biochemists, physiologists and specialists in nutrition and vitamins, not only from the point of view of experimental research but also from that of clinical practice.

BERNARDO A. HOUSSAY, Buenos Aires, Argentina

The Chemical Activities of Bacteria. By E. F. GALE, member of the scientific staff of the Medical Research Council, Cambridge, England. University Tutorial Press, London; Academic Press, New York, 1947. 199 pp. Price \$3.00.

This little book gives an excellent survey of present knowledge of the chemical activities of bacteria. Special emphasis is placed upon the role of enzymes in synthetic and

catabolic reactions and upon the influence of environmental conditions upon enzyme formation. However, numerous other topics, such as nutrition and nutritional antagonism, bacterial polysaccharides, the role of bacteria in the nitrogen cycle, the chemical basis of pathogenicity and practical methods of growing and handling bacteria for enzyme studies are also briefly discussed.

The book is intended primarily as an introduction to the subject of bacterial metabolism for students of bacteriology and biochemistry and for research workers in related fields. The treatment is relatively elementary, assuming only a knowledge of organic chemistry. The subject matter is carefully organized and clearly presented with the object of emphasizing general principles whenever possible. The text is well illustrated by numerous chemical equations, structural formulae, tables and graphs, the latter being mostly diagrammatic in character. Relatively few original data are presented and the only references are to recent reviews and books.

In so short a book covering a large and diverse field, it is inevitable that some topics should be treated rather superficially. The least satisfactory chapter is that entitled "The Nature and Identification of Bacteria," which attempts to cover the morphology, taxonomy, and cultural characteristics of bacteria in 12 pages. The reviewer feels that it would have been better to omit this chapter entirely and to refer the reader instead to standard textbooks in bacteriology for this information.

Another possible criticism is that a disproportionate amount of attention is devoted to the chemical activities of a relatively few bacteria among which *E. coli* is most prominent. The activities of numerous other bacteria of equal interest are discussed only very briefly.

In spite of these minor criticisms, the reviewer feels that Gale's book is very well written and will be most useful for students and others who are interested in bacterial biochemistry.

H. A. BARKER, Berkeley, Calif.

Advances in Enzymology, Vol. VII. Edited by F. F. NORD, Fordham University, New York, N. Y. Interscience Publishers, Inc., New York, N. Y., 1947. xi + 665 pp. Price \$8.75.

These annual reviews, the seventh volume of which has now appeared, have become almost indispensable for the progress of our science in the English-speaking countries. Because of this special usefulness a serious development should be recognized in time and checked by the editor. The annual volumes, which in the first five years contained 300-400 pages and articles 20-40 pages long, were increased in Volume VI to 563 pages, and in the present volume to 665 pages. This is due partly to the increase in length of the articles which, because of this length, lose some of their usefulness.

The most extensive article is that of G. Hevesy on "Some Applications of Radioactive Indicators in Turnover Studies," which is nearly 100 pages long. Close reading reveals that this survey of a very timely and important subject is overburdened with experimental details and contains about 60 tables. In spite of this, it tells an interesting story. It covers only the "pre-atomic age." No work on C^{14} is discussed, but only work on P^{32} (about 80 pages) and shorter sections on S^{35} and I^{131} . In the work with P^{32} , it is no longer the acid-soluble P, but nucleic acid and lipid P which are the focus of interest. The enormous turnover rate of desoxyribonucleic acid in cell nuclei

is dramatically demonstrated by studies of leucemia. A rapid regeneration of choline-containing phosphatides is also conspicuous, especially in liver. With labeled S, work on methionine and thiamine is outstanding. With radioactive iodine, biosynthesis of thyroxine *via* diiodotyrosine has been definitely established.

Two very interesting and well-written articles deal with hematin compounds: one by Hugo Theorell, "Heme-linked Groups and Mode of Action of Some Hemoproteins," and the other by S. Granick and H. Gilder, "Distribution, Structure, and Properties of the Tetrapyrroles." If the overlapping of the latter review with that of Theorell had been avoided, some pages could have been left out.

Theorell deals with the 4 pure substances: hemoglobin, cytochrome c, peroxidase, and catalase. The term "heme-linked" groups signifies the specific parts of the protein molecule which are directly bound to the heme, either by way of Fe or with the side chains of the porphyrin. The Fe-porphyrin rests as a flat disc on the protein, and two covalent bonds emerge from the Fe at right angles to the disc; either one or both of these being linked to the protein. In catalase and peroxidase, one of these bonds is an —OH group. In the case of hemoglobin, the imidazole group of histidine is "heme-linked." In cytochrome, apparently two imidazole groups are heme-linked, which would explain the inaccessibility of oxygen and CO to the Fe. The heme-linked groups in peroxidase and catalase are not known.

The paper of Granick and Gilder discusses, on the basis of Hans Fischer's ferrous protoporphin IX, the bearing of the side chains, as well as biological synthesis and decomposition. The attachment of oxygen to ferrous hemoglobin—oxygenation rather than oxidation—is explained by the stabilizing influence of the electronic configuration: the unpaired electrons of oxygen and ferrous iron became paired. In cytochrome c, the vinyl side chain of the porphyrin is linked to cysteine. From the study of different hemoporphyrins, it is found that the propionic acid side chain is necessary for combination with protein. The decomposition of the porphyrin to bile salt is apparently an autocatalytic reaction by a peroxidase formed from ferric-heme plus H_2O_2 .

Of the other articles, special mention should be made of that of Frank H. Johnson, "Bacterial Luminescence," Claude Fromageot, "Oxidation of Organic Sulfur in Animals," and Otto Schales, "Kidney Enzymes and Essential Hypertension."

F. Johnson sums up mostly the work of the school of Newton Harvey: stable luciferin in presence of the enzyme, luciferase, emits light by an oxidative reaction, the intensity of the light being proportional to the velocity of oxidation. Oxyluciferin can be partly reduced chemically and again emit light in the presence of luciferase. These facts were found with *Cypridina*, but apparently hold also for bacteria, although the system is not separable from the cell. Most of the article is concerned with the physical chemistry of this reaction. The wave length of maximal luminescence is 4,750 Å and represents per mol quantum a free energy change of —60,700 cal. This is apparently provided by oxidation of glucose, which would liberate an average of —57,340 cal. for every 2 H oxidized.

C. Fromageot deals ably with the metabolism of methionine, homocysteine, cysteine, cystine, and elementary sulfur. Many different reaction types occur: demethylation, deamination, decarboxylation, oxidation, *etc.* There is also a specific desulfhydrase, which splits S from cysteine and a transsulfuration in which homo-

cysteine reacts with serine (du Vigneaud). Because of this by-pass not all cysteine is oxidized by way of cystine. The S finally appears as sulfate ion, H_2S , or as taurine.

O. Schales describes the splendid work, particularly in this country, which correlates the essential hypertension of renal origin with the pressor substance, renin, discovered 50 years ago by Tigerstedt in Finland. It was found that renin is an enzyme which splits off the polypeptide "hypertensin" or "angiotonin" from pseudoglobulin in serum. Hypertensin, which is a vasoconstricting agent, can be destroyed by another enzyme, hypertensinase. Although the humoral origin of human renal hypertension is an established fact, the aforementioned experimental findings cannot be immediately applied to it. Especially in the chronic phase of the disease, no increased renin is found in the blood. The author also deals with the natural amines formed in the kidney, some of which gave vasoconstriction, and some vasodilation.

The following review articles, also contained in this volume, can be mentioned only briefly here:

"Permeability and Enzyme Reactions" by S. C. Brooks is not in all parts satisfactory. The author states repeatedly that the fermentation rate of living yeast may depend on permeability to sugar, but this seems highly improbable since it is independent of concentration.

"The Properties of Protoplasm, with Special Reference to the Influence of Enzymic Reactions" by William Seifriz is an interesting survey which has only a loose connection with enzyme chemistry.

"Recent Views on Asymmetric Synthesis and Related Processes" by Patrick D. Ritchie describes a field of research which was inaugurated with great expectations 40 years ago (Bredig and Fajans), but which has made little progress since. Perhaps the closest approximation to the asymmetric catalysis by enzymes is the finding (W. Kuhn) that racemates can be resolved by selective adsorption on wool fibers.

The article of Henry McIlwain, "Interrelations in Microorganisms between Growth and the Metabolism of Vitamin-like Substances," deals with the roles of nicotinamide and pantothenic acid for the growth of bacteria. The need of cozymase (coenzyme I) by some bacteria, and its destruction during fermentation, is dealt with in some detail. A similar destruction of pantothenic acid during oxidation of glucose is also correlated with the metabolic turnover.

Frederick Kavanagh, "Antibacterial Substances from Fungi and Green Plants," gives a short description of 39 antibacterial substances. The mode of action is unknown for practically all of them.

F. M. Hildebrandt, "Recent Progress in Industrial Fermentation," deals mainly with the connection of this industry with the war effort. Butanol-acetone fermentation, ethyl alcohol fermentation (including production of glycerine), and fermentation of 2, 3-butylene glycol are described from the industrial and economic viewpoints.

OTTO MEYERHOF, Philadelphia, Penna.

Les Inositols, Chimie et Biochimie. By PAUL FLEURY, Faculté de Pharmacie de Paris, and PAUL BALATRE, Faculté de Médecin et de Pharmacie de Lille. Masson et Cie., Paris, 1947 Price 300 fr.

This publication appears at a most opportune moment, as general interest has developed in both the chemistry and the biochemistry of these compounds. It happens

that almost simultaneously an English article on the Chemistry and Configuration of the Cyclitols, written from the standpoint of the sugar chemist, by Hewitt G. Fletcher, Jr. (National Institute of Health, U. S. Public Health Service, Bethesda, Maryland), has been published in the *Advances in Carbohydrate Chemistry*, Academic Press, N. Y., 1948.

These papers complement each other most fortunately. The French book gives a comprehensive description of the physical properties, structure, configuration, chemical properties, analytical characteristics, methods of determination—both chemical and biological, and a complete description of all the derivatives of *meso*-inositol and its isomers.

The chemist is pleased to find here, a critical compilation of all the necessary data for scientific work, which, otherwise, is so widely distributed throughout the literature. The reactions leading to the determination of the constitution of the inositols are well represented and adequately illustrated by formulae. There is one misleading misprint, on p. 19, where the end product of the reaction chain, formula correct, has been labeled "acide DL-talomucique" instead of DL-idosaccharic acid.

The section relating to the chemistry of inositols is excellent and up-to-date, and the same holds true for the second part, namely, Biochemistry of the Inositols. The distribution of *meso*-inositol in the plant and animal kingdom is fully described, and there is a clear and critical survey of the role of the compound in animal physiology and in the metabolism of bacteria.

A further chapter deals with inositol as a factor for the growth of yeast, and its marked specificity in this respect. Also, the interesting aspects of *meso*-inositol as a member of the B group of vitamins are carefully considered.

The publication is well indexed and can be recommended to all who are interested in the chemistry or biochemistry of the inositols. The modest price of 300 fr. (approx. \$1.00 U. S.) is also a recommending factor.

HERMANN O. L. FISCHER, Toronto, Canada

Life: Its Nature and Origin. By JEROME ALEXANDER. Reinhold Pub. Corp., New York, N. Y., 1948. Price \$5.00.

On the whole, this is a well written and interesting volume, especially the main body of the book (Ch. II–X) where the author, a chemist, deals with the proven and probable roles of the *catalysts and catalysis* in the origin of life, in the evolution of life, and in the living processes (in health and in disease) of plants and animals. These chapters will interest all mature and informed citizens. Ch. I (*How did Life Originate?*) and XII (*Philosophy, The Guide of Mental Life*) are too mixed with poetry, mysticism, and the guesses of the past, ignorant of science, to measure up to the rest of the book. The author appears to accept the dualism of mind and matter, without clearly defining his conception of the nature of mind in living matter or separate from living matter. He also appears to accept the Spencerian dictum of the unknowable: "both material and psychic ultimates are beyond the range of human conception" (p. 273).

A. J. CARLSON, Chicago, Ill.

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The Inactivation of Insulin by Tissue Extracts.

I. The Distribution and Properties of Insulin Inactivating Extracts (Insulinase) ¹

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INTRODUCTION

Although it is generally acknowledged that insulin is destroyed rapidly in the intact organism (1,2), the site and mechanism of insulin destruction is unknown. The possibility that animal tissues can inactivate insulin *in vitro* is suggested by the studies of Schmidt and Saatchian (3) and by Lehmann and Schlossman (4). The former observed that brei prepared from various tissues of the rabbit destroyed insulin during incubation. They did not investigate the nature of the system responsible for the inactivation. Lehmann and Schlossman, in a preliminary note, reported that extracts of rabbit muscle contained two active systems each of which could destroy insulin independently of the presence of the other; one system was described as a thermostable, dialyzable constituent and was believed by them to be a sulfhydryl compound of low molecular weight, whereas the other was described as a thermolabile, nondialyzable factor.

In view of the obvious importance of obtaining more information concerning the mechanism of the destruction of insulin in tissues, we have studied the ability of various tissue extracts to inactivate insulin during *in vitro* incubation.

¹ Aided in part by a grant from the Research Grants Division of the U. S. Public Health Service.

Presented in part before the American Physiological Society, Atlantic City, New Jersey, March 1948.

METHODS

Extracts were prepared from tissues immediately after their removal from an animal sacrificed by a blow on the head or by the intravenous injection of air. As much blood as possible was drained from the carcass of the animal before removal of the tissues. The extracts were prepared by homogenizing the tissues in a Waring Blender with 3 volumes of ice water. Such homogenates were centrifuged at high speed and the filtered supernatant fluids were tested for their ability to inactivate insulin. These extracts varied in their reaction from pH 6.2 to 6.8 and were adjusted to the desired pH before testing. Unless otherwise noted, all extracts were used immediately after preparation.

In a few instances the inactivation of insulin by various body fluids was tested. In such cases, the fluids or their fractions were used immediately after preparation.

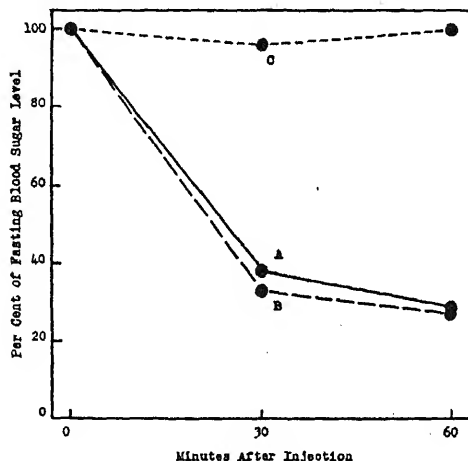


FIG. 1. The effect of rat liver extract on the activity of insulin.

A—insulin + saline; B—insulin + heat-inactivated rat liver extract; C—insulin + rat liver extract. Incubation at 37°C. and pH 7.5 for 1 hr. In each instance, the rabbits were injected with a volume of the incubation mixture which contained 4 units of insulin prior to incubation. The pooled percentages of the initial blood sugar level are A-67, B-60, C-196.

The destruction of insulin during incubation with tissue extracts or body fluids was estimated by the effect of the incubation mixture on the blood sugar of rabbits after its intravenous injection. In the standard procedure, the incubation mixture consisted of a total volume of 2 ml., containing 1 ml. of extract and eight units of insulin. After the completion of incubation, one-half of this volume, *i.e.*, 1 ml., was injected intravenously into a fasted rabbit. If no destruction of insulin had occurred, this would have been equivalent to the injection of four units of insulin, an amount of insulin which produces a profound hypoglycemia in the rabbit. Blood samples were drawn

prior to and at 30-60 min. after the intravenous injection of the incubation mixture. The change in the blood sugar concentration was expressed as the percentage of the average of two fasting blood sugar levels determined prior to injection. The sum of the percentages of the 30 and 60 min. blood samples was designated as the "pooled percentage of initial blood sugar," which in turn was utilized as a semiquantitative procedure to estimate activity. It is apparent that, if no insulin were destroyed during incubation, the "pooled percentage" would be very low. On the other hand, if all the insulin were destroyed, the blood sugar level would remain unchanged and the "pooled percentage" would equal 200 (Fig. 1).

The insulin used in this study consisted of a purified amorphous sample which assayed about 22 units/mg.² Unless otherwise noted, all experimental animals which served as the source of the tissues studied were maintained on ordinary laboratory rations fed *ad libitum*.

RESULTS

Systems capable of inactivating insulin during *in vitro* incubation were found to be widely distributed. In the rat the greatest activity per g. of tissue was found in liver extracts. Extracts prepared from kidney and muscle were found to contain progressively less activity (Table I).

TABLE I
*Distribution and Activity of Insulin-Inactivating System
in Rat Tissue Extracts*

Incubation for 60 min. at pH 7.5 and 37°C. Organ extracts prepared in usual manner. Washed blood cells resuspended in saline in original concentration. 1 ml. of insulin containing 8 units.

Tissue	Pooled percentages of initial blood sugar
Liver	211
Kidney	150
Muscle	112
Brain	84
Whole blood	92
Plasma	55
Cells	91

In general, these results substantiate the findings of Schmidt and Saatchian in reference to the activity of brei prepared from various tissues of the rabbit (3). Of other rat tissues, whole blood, plasma, and either the washed or hemolyzed cellular constituents of the blood failed to produce appreciable inactivation. Brain extracts were also relatively inactive.

² We are indebted to Dr. F. B. Peck of the Eli Lilly Company for generous supplies of insulin.

The liver from the rabbit, steer, chicken, and man (obtained both from biopsy and autopsy sources), was also found to be rich in the insulin-inactivating system. However, the extracts prepared in a similar manner from rabbit muscle and rabbit liver showed somewhat less activity than did extracts from the corresponding tissues in the rat.

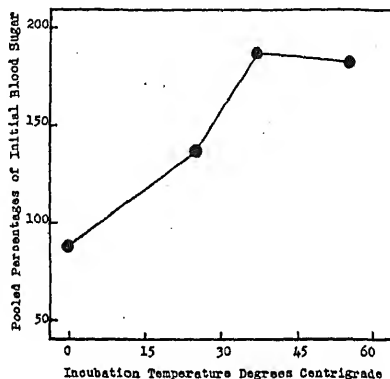


FIG. 2. The effect of incubation temperature on the inactivation of insulin by rat liver extract.

Incubation at pH 7.5 for 30 min. according to standard procedure described in text.

The system responsible for the inactivation of insulin appears to have the properties of an enzyme. It is heat-labile, being destroyed by exposure to 80°C. for 10 min. Maximum activity during incubation has been noted at reactions varying from pH 6.5 to pH 9.5. At pH 7.5, the rate of inactivation of insulin increases appreciably with a rise in the temperature of incubation from 0° to 37°C. A further increase in the incubation temperature to 55°C. fails to affect the rate of inactivation any more (Fig. 2).

In rat liver extracts, the activity was almost quantitatively removed from solution by precipitation of the proteins, either through adjustment of the reaction to pH 5 or by half-saturation with ammonium sulfate at pH 6.7. During precipitation, only moderate destruction of the active proteins occurred and much of the activity was recovered from the washed precipitates by redissolving the latter with adjustment to pH 7.5-8.0.

At pH 7.5 and 37°C. the destruction of insulin proceeds at a fairly rapid and easily measurable rate which gradually decreases towards

the end of incubation (Fig. 3). Rat liver extracts have been obtained with such an activity that 1 ml. can destroy as much as 8 units of insulin during a 30 min. incubation period. The effect of varying the amount of insulin during incubation is also illustrated in Fig. 3, which indicates the degree of destruction of varying amounts of insulin by a rat liver extract during a 30 min. period of incubation at pH 7.5 and 37°C.

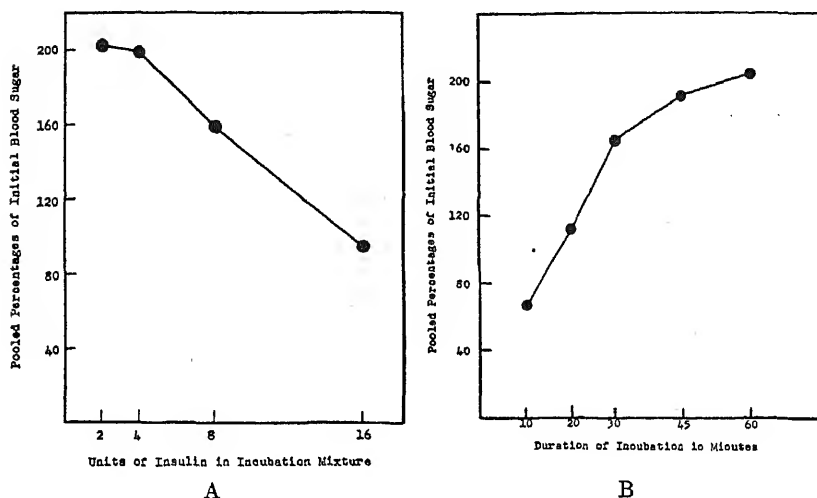


FIG. 3. Effect of duration of incubation and quantity of insulin added to incubation mixture on the destruction of insulin by rat liver extract.

In both "A" and "B," mixtures were incubated at 37°C. and pH 7.5. The rabbits were injected with a volume of mixture which prior to incubation contained one-half the total quantity of insulin originally present in the incubation mixture. In "A" the incubation mixture contained 8 units.

The effect of dialysis on the activity of its liver preparations was studied. For this purpose extracts were dialyzed for variable periods of time in Visking dialysis sacs against frequent changes of ice-cold distilled water. This operation was performed in the cold room. At the end of the period of dialysis, the dialyzable substances in the external medium were shell frozen and concentrated by lyophilization. The dried products were reconstituted to the original volume of the whole extract or to a fraction thereof and readjusted to pH 7.5. In no case did such concentrated dialyzates show any activity in the destruction of insulin (Table II).

TABLE II

Effect of Dialysis on Inactivation of Insulin by Rat Liver Extract

Type of extract	Pooled percentage initial blood sugar
Before dialysis ("A")	194
"A" boiled 10 min. ("B")	79
"A" after dialysis ("C")	91
Concentrate of dialyzate ("D") of "A"	84
"C" + "D"	125
"D" boiled 10 min. + "C"	118
"B" + "C"	125

Incubation at pH 7.5 and 37°C. for 60 min.

In all instances, dialysis of rat liver extracts for 3 hr. resulted in a decrease in their ability to inactivate insulin. The activity of the nondialyzable residue of such extracts could often be restored partially through the addition of certain metallic ions. Table III illustrates the effect of the addition of such ions to a dialyzed extract and indicates the ability of manganese and magnesium ions to restore activity. Other ions that were tested were found to be ineffective in this regard.

TABLE III

Effect of Metallic Ions on Activity of Dialyzed Rat Liver Extract

Extract	Inorganic ions added	Pooled percentage initial blood sugar
Before dialysis		195
After dialysis		95
After dialysis	K ⁺	124
After dialysis	Mn ⁺⁺	180
After dialysis	Mg ⁺⁺	167
After dialysis	Cu ⁺⁺	57
After dialysis	Zn ⁺⁺	64

Incubation at pH 7.5 and 37°C. for 45 min. Inorganic ions added to a final concentration of 9×10^{-4} M.

The thermostability of the whole tissue extract can be attributed to the properties of the nondialyzable fraction since the addition of manganese ions failed to restore activity to a heat-inactivated nondialyzable rat liver extract fraction. Attempts to increase the activity of a dialyzed rat liver by addition of the concentrated dialyzable fraction to the nondialyzable material have not been successful. Such efforts resulted in consistent but very slight increases of activity, but complete activity was never restored. The results of the various effects of dialysis are illustrated in Tables II and III.

When dialysis of rat liver extract was continued for more than 3 hr., a permanent loss of activity ensued which could not be restored through the addition of manganese or magnesium ions. It is of interest to note that even prolonged dialysis of beef or rabbit liver extracts did not result in a diminution of the insulin-inactivating ability of such extracts.

The inactivation of insulin by rat liver extracts could be inhibited by a number of substances. The data in Table IV illustrate the effect of the addition of various substances on the activity of rat liver extract incubated with 8 units of insulin at 30 C. and pH 7.5. Thus, the *in vitro* addition of small quantities of copper, zinc, and certain other heavy metals, resulted in an almost complete suppression of the previous ability of the system to destroy insulin. On the other hand, salts of magnesium, manganese, sodium, and calcium, failed to influence the activity of the whole system. Moreover, the activity was found to be markedly inhibited by 10^{-3} M iodoacetate and iodosobenzoate.

TABLE IV

Effect of Inhibitors on Destruction of Insulin by Rat Liver Extracts

Incubation for 30 min. at pH 7.5 and 37°C. Standard procedure as described in text. Incubation mixtures originally contained 8 units of insulin.

Inhibitor	Final concentration	Pooled percentages of initial blood sugar
None		195
CuSO ₄	0.0005 M	91
ZnSO ₄	0.0005 M	118
Iodoacetate	0.001 M	117
Iodosobenzoate	0.001 M	84
Soybean inhibitor	0.45 mg.	212
Normal human serum	0.9 ml.	195

To determine whether the inactivation of insulin by liver extracts is due to some nonspecific proteolytic enzyme system, the effect of such extracts on denatured hemoglobin at pH 7.5 was measured. No appreciable effect was noted. Furthermore, the addition of the relatively nonspecific proteinase inhibitors of the soybean and of human serum did not appreciably decrease the insulin-inactivating properties of the extracts (Table IV).

DISCUSSION

The inhibition of the activity of the insulin-destroying system in tissue extracts by metals such as copper, and by organic compounds

such as iodoacetate and iodosobenzoate, may suggest the necessity for the participation of a sulfhydryl group in the activity of the system. Attempts to obtain further evidence to substantiate this hypothesis have been hampered by our inability to reactivate, by cysteine, extracts inhibited in such a manner. Even if the activity of this system does depend upon the participation of a sulfhydryl group, the system nevertheless appears to resemble a true enzyme rather than a sulfhydryl compound of low molecular weight such as that reported by Lehmann and Schlossmann (4). The deduction of its enzyme-like nature is based upon its heat-lability, its high molecular weight (as indicated by its nondialyzable nature), its association with a protein function of the extract, the kinetics of its reaction with insulin, and its absence from brain and blood, both of which are relatively rich in sulfhydryl compounds of low molecular weight. Furthermore, the apparent role of manganese and magnesium ions as activators conforms to what is known about the similar behavior of certain other enzymes.

The nature of the method of destruction of insulin by the tissue extracts has not yet been demonstrated. Schmidt and Saatchian (3) assumed that minced rabbit tissues destroyed insulin by virtue of the proteolytic activity believed to be present in their preparations. That such an explanation can account for the inactivation of insulin by the tissue extracts used in this study does not appear to be the case. In the first place, slightly alkaline extracts of rat liver do not demonstrate appreciable proteolytic activity at pH 7.5 as measured by their ability to release tyrosine-like substances from a denatured hemoglobin substrate. The insulin-destroying activity was found not to be inhibited by the addition of the relatively nonspecific protease inhibitors of the soybean, although these latter are known to inhibit the proteolytic substances which are active at neutral or slightly alkaline reactions (Table IV). Whereas such evidence does not prove conclusively the lack of the proteolytic nature of the insulin inactivator, its properties are such as to render it improbable that it resembles the trypsinases or cathepsins. Accordingly, pending further investigation, it is proposed for the sake of convenience to characterize the active principle in these extracts as "insulinase."

From the preceding, there seems to be but little doubt that tissues do contain systems capable of destroying insulin at a rather rapid rate. Indeed the rate of their *in vitro* activity is far greater than necessary to

account for the destruction of all the insulin assumed to be secreted by the pancreas during the course of a day. Whether or not the tissue insulinases play an actual role in the intact organism in the destruction of insulin is under study at the present time.

SUMMARY AND CONCLUSIONS

1. A system has been found in extracts of various tissues of different animal species which appears to be an enzyme capable of destroying the physiological activity of insulin during *in vitro* incubation. This system is tentatively designated as insulinase.

2. The distribution of insulinase in various tissues and species and its properties have been noted.

3. Evidence has been adduced to indicate that insulinase does not resemble ordinary proteolytic enzymes. It is suggested that the activity of insulinase may depend upon the participation of a sulphydryl group.

4. The apparent role of magnesium and manganese salts, and of zinc, copper, and other metals, as activators or inhibitors of insulinase has been described.

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The Inactivation of Insulin by Tissue Extracts.

II. The Effect of Fasting on the Insulinase Content of Rat Liver

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INTRODUCTION

In a previous communication the distribution and properties of the insulin-inactivating system (insulinase) of rat tissues were described (1). Although this system actively destroys insulin *in vitro*, it became pertinent to determine whether or not it plays any role in the regulation of carbohydrate metabolism in the intact animal. Toward that end, we have investigated the influence of a variety of factors on the activity of insulinase. As a first step in that direction, we studied the influence of fasting, since the deprivation of food is known to be associated with profound changes in mammalian metabolism.

METHODS

The insulinase activity of liver extracts prepared in a standardized manner was assayed according to a uniform procedure. Albino male adult rats, weighing from 200 to 300 g., were stunned by a blow on the head and their bodies drained of blood by severance of the great vessels in the neck. The liver was rapidly removed, freed from adjacent tissues, weighed and blended immediately with 3 volumes of ice cold water in the Waring Blender for exactly 2 min. The resultant homogenates were centrifuged in the cold room at high speed for 5 min. and the supernatant fluid decanted through gauze. The extracts so obtained were adjusted to pH 7.5 by the addition of the required number of drops of *N* NaOH. With this procedure, each ml. of extract is equivalent to 0.3 g. of liver. All extracts were used immediately and were incubated in a

¹ Aided in part by a grant from the Research Grants Division of the U. S. Public Health Service.

total volume of 2 ml. containing 8 units of amorphous insulin² and 1 ml. of extract.

The mixtures were incubated for 15 and 30 min., and the destruction for insulin in each mixture was estimated by determination of the effect on the blood sugar level of rabbits. In the standard procedure, 1 ml. of the incubation mixture, which had contained 4 units of insulin before incubation, was injected intravenously into fasted rabbits and the deviations of the 30 and 60 min. blood sugar level from the preinjection level were expressed as the "pooled percentage of the initial blood sugar level." This serves as a semiquantitative index of the insulin that remains in the mixture and thereby of the amount of insulin that was destroyed during incubation (1).

Prior to fasting, all animals had been kept on a stock laboratory diet (Purina Laboratory Chow Checkers) and fed *ad libitum*. During the period of deprivation of food, the rats were supplied only with water. In some experiments, after a period of starvation, the animals were refed with the same stock laboratory diet.

RESULTS

The effects of fasting and subsequent refeeding on the insulinase activity of extracts equivalent to comparable quantities of liver are summarized in Table I and illustrated graphically in Fig. 1.

TABLE I
Effect of Fasting on the Insulinase Content of Rat Liver

Duration of fast	Number of rats	Pre-fasting weight	Loss in weight	Liver weight	Blood sugar	Insulinase activity ^a		Significance of differences ^b "t" values	
						15 min. incubation	30 min. incubation	15 min. incubation	30 min. incubation
0	24	g.	g.	g.	mg.-%	139±43.0	179±35.7	—	—
24	4	219	0	8.2	96	132±41.8	171±48.5	0.213	0.212
48	6	273	7	8.0	82	91±18.0	121±50.2	1.881	2.063 ^c
72	6	257	26	6.3	75	69± 8.3	97±32.0	2.918 ^d	3.748 ^d
96	6	263	35	6.5	69	82±33.2	107±37.9	2.710 ^c	3.674 ^d
162	10	245	32	5.8	86	62±12.5	105±23.6	3.140 ^d	3.202 ^d
162	4	207	54	4.9	103				
Refed 24 hr. after 96 hr. fast	9	248	1	10.3	121	122±39.7	161±42.9	0.724	0.850

^a Insulinase activity is expressed in terms of the pooled percentage of the initial blood sugar of rabbits injected with incubation mixture (see text).

^b Significance of differences calculated in terms of Fisher's "t" values (5).

^c Difference is significant at 5% level.

^d Difference is significant at 1% level.

² We are indebted to Dr. F. B. Peck of the Eli Lilly Co. for generous supplies of amorphous insulin assaying about 22 units/mg.

It is evident that fasting is associated with a marked reduction in the insulinase activity of extracts from each gram of liver. The data reveal that the reduction in insulinase activity first approaches statistical significance at the end of 48 hr. of fasting and remains significantly decreased for as long as 162 hr., which represents the maximum observed duration of starvation. When animals which had been fasted for 96 hr. were permitted to feed *ad libitum* for 24 hr., the insulinase activity of their liver extracts was restored to the range of values obtained from unfasted rats.

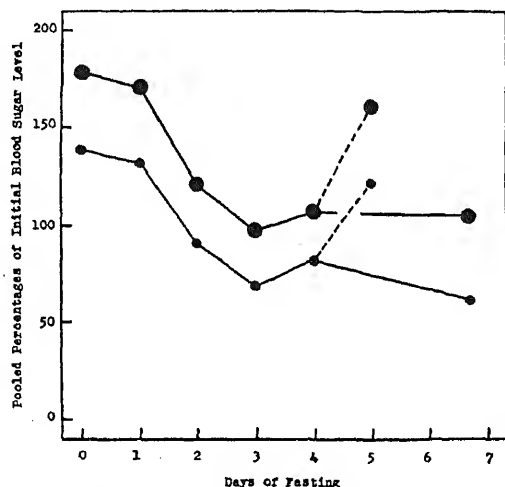


FIG. 1. The effect of fasting and of refeeding on the insulinase activity of rat livers. ●—● represents the degree of insulin inactivation by extracts incubated at 37°C. and pH 7.5 for 30 min. ○—○ represents the degree of insulin inactivation by extracts incubated for 15 min. --- represents the effect of 24 hr. refeeding of rats which were fasted for 4 days.

DISCUSSION

It is evident that fasting is associated with a marked reduction in the insulinase activity of liver extracts of adult male rats. The fact that a reduction in both body weight and liver weight occur at the same time indicates that the decreased insulinase activity per ml. of extract (0.3 g. of tissue) represents a real decrease in the total insulinase content of the liver. It is difficult to assess the physiological significance of this decrease, since there are very little pertinent data

available concerning the effects of starvation on the metabolism of carbohydrates in the rat.

Selye (2) has described a decrease in the blood sugar level of the rat during the first 48-72 hr. of fasting. As the duration of fasting was prolonged beyond this period, he noted a steady increase in the blood sugar with the eventual production of a moderate hyperglycemia. Coincident with these changes, he noted an increased sensitivity to injected insulin during the first 48 hr. of the fast followed by the development of a decreased sensitivity to exogenous insulin as the period of starvation was prolonged. However, our own experience is not in accord with the above. Utilizing the percentage decrease of the blood sugar level following a single injection of a test dose of 0.5 units/kg. of body height, we could find no evidence of a significant change in the sensitivity of the rat to insulin throughout a fast of as long as 120 hr. Furthermore, in these same animals a prolonged fast was associated with a relatively minor decrease in the blood sugar level and at no time with an increase of significant proportions.

The studies of Best and his colleagues (3) have demonstrated that fasting is accompanied by a marked decrease in the insulin content of the rat pancreas and that the content can be restored to the normal level by feeding a balanced diet. If the insulin content of the pancreas is an accurate index of the amount of insulin available to the organism, it could be anticipated that the decrease in the pancreatic insulin content coincident with fasting would result in insulin insufficiency and a consequent hyperglycemia. Apparently, hyperglycemia was not observed by Best *et al.* nor could we find any indication of hyperglycemia in our own fasted rats, since the 162 hr. fasted rats had an average blood sugar of 103 which is within the normal range.

The apparent paradox of insulin insufficiency without an associated hyperglycemia may be explained in view of our own observations. If insulin is destroyed in the intact animal as the result of its inactivation of insulinase, the quantity of insulin required for the maintenance of carbohydrate metabolism at a normal level might be expressed *not* in terms of the amount secreted by the pancreas but rather as the balance between the amount secreted and the amount destroyed by insulinase. If this is true, it can be postulated that any decrease in insulinase activity would result in a decrease in the amount of insulin secretion neces-

sary for the maintenance of the blood sugar at its normal level. This hypothesis is strengthened by the observation that the administration of exogenous insulin results in a reduction of the insulin content of the rat pancreas (4). Presumably, the supply of exogenous insulin so increases the total amount of available insulin as to require a lower rate of secretion by the pancreas in order to maintain the supply of available insulin at its normal level.

Until further data are obtained concerning the status of carbohydrate metabolism in the fasted rat, it is inadvisable to conjecture further about the significance of the decrease of liver insulinase in that animal. All that can be stated conclusively at the present time is that fasting induces both a decrease in the insulin content of the pancreas of the rat (3), and a simultaneous decrease in the liver content of a system which is capable of destroying insulin. Further, the feeding of a balanced diet causes a restitution of the insulin content of the pancreas (3) and likewise a restitution of the insulinase content of the liver.

SUMMARY

Fasting produces a marked reduction in the insulin-inactivating system (insulinase) of rat livers.

The insulinase activity of the fasted rat liver is restored to normal by feeding a balanced diet.

It is postulated that there may be some relationship between the insulin content of the pancreas and the insulinase activity of the liver.

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The Prevention of Insulin Hypoglycemia by DL-Glyceraldehyde

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INTRODUCTION

A number of workers, using brei or extracts of various tissues, have shown that in the presence of glyceraldehyde the formation of lactic acid from carbohydrate is greatly diminished or entirely prevented (1, 2, 3). The nature of this inhibition in the carbohydrate cycle apparently depends on whether the dimeric or the monomeric form of the aldehyde is used. The dimeric form has been found to inhibit the esterification of both glycogen and glucose with phosphate (4). Monomeric glyceraldehyde exhibits no effect on the conversion of glycogen to the Cori ester but exerts its inhibitory action on the primary esterification of glucose to glucose-6-phosphate. The specificity of action of the monomeric form on the hexokinase system has been reported by Stickland (5) who obtained reversal of the inhibition by adding yeast hexokinase to the muscle extracts. Süllman (6) found no inhibition of lactic acid formation by glyceraldehyde from glucose-6-phosphate.

This demonstration of a "specific" inhibition of the primary esterification of glucose by monomeric glyceraldehyde *in vitro* suggested that an investigation be made of the action of the aldehyde administered to the intact animal with and without added insulin.

MATERIALS AND METHODS

In the preliminary study herein reported rats, dogs, cats, and rabbits were used as test animals. All animals were in a fasting condition when the tests were made and were injected as follows: *Group 1*, glyceraldehyde; *Group 2*, insulin; *Group 3*, glyceraldehyde and insulin; *Group 4*, glucose; and *Group 5*, glucose and insulin.

The glyceraldehyde was given by continuous intravenous injection at the rate of 1 g./kg. body weight/hr., in a 5% solution, for 1 hr. This was preceded by an initial priming dose of 2 cc. of 5% glyceraldehyde because of the rapidity with which the

glyceraldehyde was removed from the blood. The insulin was injected subcutaneously in amounts of 5 units/100 g. weight in the rats and 5 units/kg. weight in other animals. In the groups receiving both insulin and glyceraldehyde, the insulin was injected at the start of the glyceraldehyde infusions. The glucose was injected by infusion of a 10% solution and at the rate of 2 g./kg. weight/hr. (the glucose injected thus being equimolar with the glyceraldehyde). The animals were anesthetized with sodium pentobarbital by intraperitoneal injection. Blood inorganic phosphate was determined by the method of Fiske and SubbaRow (7) and lactic acid by the method of LePage (8).

TABLE I

The Determination of Glyceraldehyde in Aqueous Solutions Containing Glucose

Tube (duplicate)	Contents	3 min. reduction at 70°C. (glyceraldehyde)	10 min. reduction at 100°C.	Glucose value by difference
1	Distilled water	0	0	0
2	0.5 mg. glyceraldehyde	0.390 mg.	0.388 mg.	0
3	0.5 mg. glucose	0	0.496 mg.	0.496 mg.
4	0.5 mg. glyceraldehyde 0.2 mg. glucose	0.386 mg.	0.579 mg.	0.193 mg.
5	0.5 mg. glyceraldehyde 0.4 mg. glucose	0.390 mg.	0.788 mg.	0.398 mg.
6	0.5 mg. glyceraldehyde 0.6 mg. glucose	0.382 mg.	0.987 mg.	0.605 mg.
7	0.2 mg. glyceraldehyde 0.5 mg. glucose	0.168 mg.	0.670 mg.	0.502 mg.
8	0.4 mg. glyceraldehyde 0.5 mg. glucose	0.323 mg.	0.820 mg.	0.497 mg.
9	0.6 mg. glyceraldehyde 0.5 mg. glucose	0.476 mg.	0.970 mg.	0.494 mg.

A Schering-Kahlbaum preparation of racemic glyceraldehyde was used. This was refluxed for 30 min. with redistilled acetone (20 cc. acetone to 1 g. of the aldehyde) before using. The mixture was filtered under suction and the residue well dried. The aldehyde was made up in a 5% solution and allowed to stand for 24 hr. On standing, the dimeric aldehyde resolves to the monomeric form (4).

Since reduction of the copper solution occurs with glyceraldehyde as well as with glucose it was necessary to find methods for the separate estimation of these substances in the blood filtrates. The determination of glucose in the presence of glycer-

aldehyde (Tables II and III) was accomplished by fermenting the glucose by yeast. Baker's yeast (*Saccharomyces cerevisiae*) during 30 min. of incubation in the blood filtrates at 37°C. had no action on the glyceraldehyde. By employing the yeast method for the blood sugar estimations, other nonspecific reducing substances in the blood filtrates were likewise excluded.

TABLE II

Summary of Changes in Inorganic Phosphate, Lactate, and Sugar in Blood Following the Administration of Glyceraldehyde, Glyceraldehyde and Insulin, Insulin, Glucose, and Glucose and Insulin^a

	Animals	No.	Group I		Group II		Group III		Group IV		Group V	
			Glyceraldehyde		Insulin		Glyceraldehyde and insulin		Glucose		Glucose and insulin	
			a ^b	b ^c	a	b	a	b	a	b	a	b
Blood sugar (mg.-%)	Dogs	4	82	108	89	48	86	113				
	Cats	4	85	160	84	43	87	136				
	Rats	5	77	170	98	44	80	165	111	419	90	225
	Rabbits	2	89	173	91	46	80	150	101	214	86	148
Blood inorganic phosphate (mg.-%)	Dogs	4	4.6	4.1	4.7	2.8	5.1	4.5				
	Cats	4	4.4	2.8	4.6	2.8	4.9	3.6				
	Rats	5	4.7	5.6	5.3	5.3	4.6	5.7	5.0	5.2	5.6	4.7
	Rabbits	2	5.7	3.7	4.7	4.7	3.4	4.9	5.0	4.8	5.7	4.3
Blood lactate (mg.-%)	Rats	5	17	21	16	15	18	20	17	18	16	13
	Rabbits	2	19	24	19	14	17	16	17	14	17	15

^a Glyceraldehyde administered by continuous intravenous injection of a 5% solution at a rate of 1 g./kg. body weight/hr.; glucose, by continuous intravenous injection of a 10% solution at a rate of 2 g./kg. body weight/hr., and insulin by subcutaneous injection of 5 units/100 g. body weight in rats and 5 units/kg. body weight in other species.

^b Blood sample taken prior to injection.

^c Blood sample taken 1 hr. after injection.

The method for determining the glyceraldehyde in the blood filtrates was based on the well-known fact that this substance will reduce Fehling's solution in the cold. To accelerate reduction of the copper solution by the glyceraldehyde, the blood filtrates were warmed with the copper solution for 3 min. at 70°C. and then cooled immediately.¹ The values obtained using standard test solutions are shown in Table I. Good

¹ Cf. method described by Sansum, W. D., and Woodyatt, R. T., *J. Biol. Chem.* **24**, 327 (1916).

agreement was obtained when this method was tested on blood samples to which known amounts of the glyceraldehyde were added. The values shown indicate that the reduction by glyceraldehyde of the copper solution, both at 70°C. for 3 min. and at 100°C. for 10 min., is approximately 80% of that obtained with an equal weight of glucose at 100°C. for 10 min.

RESULTS

The administration of monomeric DL-glyceraldehyde by continuous intravenous injection was found to elevate the blood sugar of the dog, cat, rat, and rabbit. When insulin was given at the start of the glyceraldehyde infusions, the reduction in the blood sugar values obtained with insulin alone did not occur. The data obtained are summarized in Table II. It was found that, although the glyceraldehyde elevated the blood sugar levels in the dog, cat, and rabbit, it depressed the levels of inorganic phosphate. In rats, on the other hand, this reduction in blood inorganic phosphate was not observed following the administration of glyceraldehyde (Table II).

TABLE III

Effect of the Liver on the Removal of Glyceraldehyde from the Blood ^a

	Rabbit no.	Blood sugar (mg.-%)	Glyceraldehyde (mg.-%)	Blood inorganic phosphate (mg.-%)	Blood lactate (mg.-%)
After 1st stage hepatectomy and before injecting glyceraldehyde	1	118	0	4.5	12
	2	124	0	4.3	19
After injecting glyceraldehyde for 30 min. and before 2nd stage hepatectomy	1	319	0	4.5	12
	2	208	20	4.0	25
After injecting glyceraldehyde for 30 min. following 2nd stage hepatectomy	1	246	319	5.2	55
	2	180	236	4.6	40

^a Glyceraldehyde administered by continuous intravenous injection at the rate of 1 g./kg. body weight/hr. in a 5% solution.

The glyceraldehyde was found to disappear very rapidly from the blood stream when infused into intact animals, but reached a high concentration in the blood when administered intravenously to 2 hepatectomized rabbits (Table III). The rabbits were hepatectomized

by the two-stage method of Drury (9). The animals were injected for 30 min. with glyceraldehyde prior to hepatectomy, and for a further 30 min. period immediately after total removal of the liver.

DISCUSSION

The findings after hepatectomy suggest that the liver is essential in effecting the removal of glyceraldehyde from the blood of the infused animal. Rosenthal (10) has shown that the liver can ferment glyceraldehyde at a significant speed, and Embden (11), and Needham and Lehmann (3), found that the liver *in vitro* rapidly converts D-glyceraldehyde to D-lactate. Needham and Lehmann also reported that the conversion of glyceraldehyde to lactic acid occurs in tissues other than the liver. In the data presented in Table III the high glyceraldehyde values do not indicate any considerable fermentation of the aldehyde extrahepatically.

Stöhr (12), Liaci (13), and Embden (14), have demonstrated hepatic glycogenesis following the administration of glyceraldehyde orally or intraperitoneally. The formation of glycogen from glyceraldehyde has also been shown by Cori (15) and by Stetten (16). Accordingly, the disappearance of the infused aldehyde from the blood of the intact animal may be accounted for by a conversion of the glyceraldehyde to sugar in the liver. This conversion may occur through the prior formation of lactic acid both intra- and extrahepatically. The increase in blood sugar may have been due partly to a toxic effect of glyceraldehyde in the liver manifested by liver glycogenolysis and resultant hyperglycemia.

Stickland (5) reported that the inhibition of yeast hexokinase in muscle extracts required a concentration of 0.003 *M* glyceraldehyde, the equivalent of 27 mg.-% in the blood. The data in Table III show that, after removal of the liver, the blood sugar values diminished despite the fact that the glyceraldehyde in the blood reached a mean concentration of approximately 275 mg.-%. The reduction in the blood sugar values in the glyceraldehyde-infused liverless animal indicates that in some extrahepatic tissues, at least, no inhibition of the hexokinase system by glyceraldehyde occurred. The explanation may be that the blood glyceraldehyde values do not reflect the concentration of aldehyde at the enzyme surface in the cell or that the optimal conditions are markedly different for inhibition of yeast hexokinase

in muscle extracts and for inhibition of hexokinase *in vivo* by the aldehyde.

In one experiment on the cat, the insulin was administered by intravenous injection together with the glyceraldehyde. The result was similar to those obtained in experiments in which insulin was given by subcutaneous injection, thus excluding the possibility that glyceraldehyde prevented the insulin hypoglycemia by a retardation or interference with insulin absorption. It would appear from this preliminary study that the hyperglycemia produced by the intravenous infusion of glyceraldehyde is largely responsible for its effect in abolishing the usual hypoglycemic response to insulin.

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SUMMARY

The injection of monomeric DL-glyceraldehyde by continuous intravenous injection resulted in a hyperglycemia and a reduction in the blood inorganic phosphate. When insulin was administered in conjunction with the glyceraldehyde, the usual marked hypoglycemic response to insulin did not occur. It is suggested that the abolition of insulin hypoglycemia by glyceraldehyde is due to a hyperglycemia produced in large part by an intrahepatic conversion of the aldehyde to glucose.

Methods are described for the estimation of DL-glyceraldehyde in the presence of blood sugar.

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The Colorimetric Determination of Lysine

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INTRODUCTION

Colorimetric methods for the determination of the basic amino acids are desirable for many studies since those involving isolation or microbiological assay are not sufficiently simple or rapid, and since the chromatographic method does not seem generally applicable at the present time. Unfortunately, unlike other amino acids of this group, lysine is not readily determined colorimetrically. In 1946 Nelson *et al.* presented a paper on the determination of lysine by the color developed with the phenol reagent after bromination (1), and this method was later developed more fully by the same workers, who substituted chlorine for bromine (2). Tyrosine, tryptophan, and other amino acids reduce the sodium tungstate-sodium molybdate reagent even after treatment with bromine, and phenylalanine and histidine behave like lysine by reacting with the reagent after bromination. It is difficult to make a preliminary separation of lysine from all of the other amino acids, including histidine, which interfere in the determination, but the method may be applied to the basic fraction of amino acids separated by permutit, since the interference by histidine can readily be corrected.

EXPERIMENTAL

A fraction of the basic amino acids which is suitable for the determination of lysine may be isolated from proteins in the following way. The protein is hydrolyzed by boiling in 6 *N* HCl for 18 hr. After dilution, the reaction mixture is adjusted to about pH 5 with NaOH, and the solution decolorized by boiling with Norit. Portions representing about 30 mg. of protein are then shaken in a volume of 25 ml. with two 1 g. portions of permutit which has been treated with 0.33 *N* NaOH, and washed with water until free of alkali. The contaminating amino acids are washed out with small portions of water, and the bases eluted from the permutit with 0.33 *N* NaOH¹ in 5 portions of 10 ml. each.

¹ A small precipitate usually appears under these conditions on subsequent addition of sodium carbonate and phenol reagent, but this may be removed by centrifugation without affecting the results.

The method adopted for the determination of lysine is as follows. Place the samples containing 0.1 to 0.7 mg. of lysine in 22 × 200 mm. test tubes graduated at 25 ml., and cool the solutions for about 15 min. in a bath of ice water. Add saturated bromine water drop by drop until colored, and then 3 drops in excess. Allow the tubes to remain in the bath for 30 min. and then remove excess bromine by aeration from the cold solutions. This may be done readily by fitting the tubes with inlet and outlet connections and absorbing the bromine in waste tubes containing 10% urea. After aeration for 10 min., remove the fittings and wash them into the tubes with 4.5 ml. of water. Transfer the tubes to a bath at 60°C. and allow the solutions to come to temperature. Add 7.5 ml. of 20% sodium carbonate which is also at 60°C. Finally, remove one tube at a time, add 2.5 ml. of phenol reagent,² mix, and cool at room temperature. Dilute to 25 ml. and read in the photometer at 680 m μ ³ in 30 min.

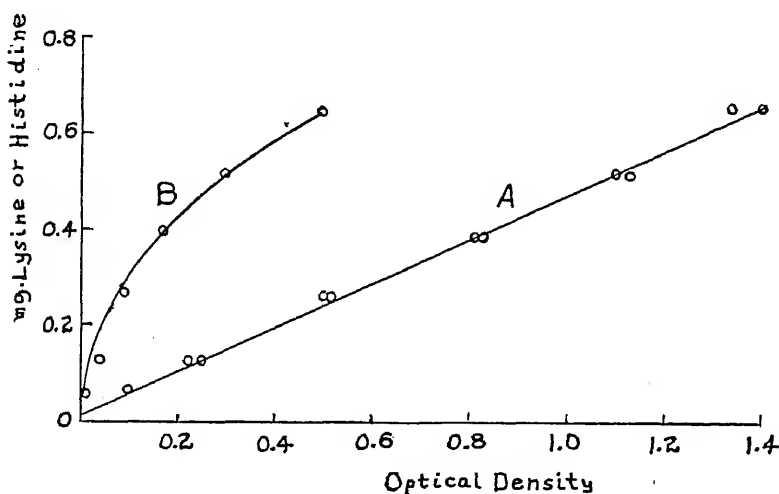


FIG. 1. The colorimetric determination of lysine. Curve A—lysine; Curve B—histidine.

In Fig. 1, curve A shows the values of optical density in a series of solutions containing 0.08–0.80 mg. of lysine hydrochloride. All the values fall on a satisfactory straight line. Although the presence of arginine or ammonium chloride does not affect the results significantly, appreciable amounts of histidine in the solutions cause elevated values.

² Prepared according to Folin and Ciocalteu (3).

³ The photometric readings were made with a Coleman Junior Spectrophotometer at 680 m μ , but satisfactory results were also obtained with filter 66 on both the Klett-Summerson and Evelyn colorimeters.

Curve B of the figure shows the values of optical density in solutions of histidine which must be subtracted to correct for the presence of this amino acid.

The method was applied to a sample of crystalline egg albumin which had been recrystallized 3 times.⁴ The values of 65.0 mg./g. of protein after correction for histidine and 67.2 mg. before correction agree quite well with those of the literature (4).

SUMMARY

A method is described for the colorimetric determination of lysine with the phenol reagent, after treatment with bromine, which may be applied to the basic amino acids separated by permutit. The presence of appreciable amounts of histidine requires correction. An analysis of crystalline egg albumin shows 65 mg. of lysine/g. of protein.

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⁴ We are indebted to Prof. R. K. Cannan for this preparation.

Some Factors Which Influence Methionine Excretion in the Rat

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INTRODUCTION

In an earlier study on medical students (1), urinary methionine excretions were determined after various dietary regimes and after giving test supplements of the free amino acid. The usual excretion was not affected by a low protein diet for 3 days, but was decreased by a high fat diet or fasting for the same period. Johnson *et al.* (2) reported the methionine excretion to be constant at different levels of protein or methionine intake, and found no evidence for the utilization in man of supplements of the free amino acid from the sulfur or nitrogen excretions. However, methionine was found by Brush *et al.* (3) to decrease urinary nitrogen losses and to spare the body tissues of the rat after deficient protein intakes, but they suggested that, under such conditions, "this amino acid does not act in the general maintenance of body tissues but in the synthesis of functional proteins and important metabolites."

These findings, as well as those of others (4,5), emphasizing the important metabolic role of methionine, and ours (1), indicating possible variations in its excretion, suggested the following questions. Can the amount of methionine in the urine be used as an indication of its utilization under varying conditions? Will an increased need for methionine be reflected by a decreased excretion after a test supplement of the free amino acid? And if there is an increased need for lipotropism by animals on high fat diets and fasting, will this be indicated by the methionine excretion when choline is supplied to spare methionine that might otherwise be used for such a purpose? This study was an attempt to collect further evidence that might aid in answering these queries.

EXPERIMENTAL

The first experiment was a study of the effect on methionine excretion of dietary methionine intake and of the injection of the free amino acid after extended periods on low and higher protein diets. Three groups of white male rats averaging 240 g. in weight were fed a commercial stock,¹ 5, and 25% protein diets. The composition of the 5% protein diet was the same as that of Diet 1 previously used (6). The higher protein diet was a similar one with a part of the starch and glucose² replaced by casein. Both of the latter diets contained 40% fat. After 22 days on these diets, 24 hr. urine samples were collected under light mineral oil from each of these animals for methionine determinations. At later dates, the latter two groups of rats were injected, intraperitoneally, with 30 and then 60 mg. of DL-methionine/100 g. body weight. One-half of these amounts was given in the morning and one-half in the afternoon to avoid excess flooding of the animal with the amino acid. The solution was injected to avoid differences in absorption that might follow oral administration with varying conditions in the intestinal tract. A similar saline injection was found not to influence the methionine excreted. The urines were collected and the methionine content determined.

The increased excretion of methionine by the animals on the stock diet suggested a more detailed investigation of the effect of variations in the protein and fat content of the diet. In a second experiment, 7 groups of 8 rats, weighing approximately 350 g., were placed on various dietary regimes for 3 days. The first group had been on the stock diet for some time. The diet of the next 5 groups varied from 5 to 50% protein and 5 to 40% fat, in each case at the expense of the carbohydrate of the diet. The last group was fasted for 3 days. Daily urine collections were obtained from each of these animals and the methionine content determined.

The study of the effect of a test supplement of methionine or choline on the urinary methionine when the animals were fed or fasted, was undertaken in the third experiment. Five groups of 8 rats, weighing approximately 315 g., were used. Urines for methionine determinations were collected for 1 day from the animals on the stock diet, and from all the others on the third day of fasting. Two groups, fed and fasted, were given intraperitoneal injections of 50 mg. of DL-methionine/100 g. body weight, divided as before into 2 equal supplements, on the day the urine was collected. Another fasting group was injected with 10 mg. of choline/100 g. body weight twice daily during the 3 day fast.

The animals received the experimental diets and distilled water *ad libitum* during these studies. The daily food intake and weight change of each animal were recorded. The methionine intake was calculated from the individual average food intakes after analysis of the stock diet, and by assuming that the casein contained 3.1% methionine (7). The urinary methionine was determined in all cases on separate urine collections from each animal, according to the method of Albanese *et al.* (8). The data were analyzed according to the *t* method of Fisher (9), and only those results showing

¹ The company supplying the widely used complete rat diet included the following analysis: 24.8% protein, 4.7% fat, and 49.3% carbohydrate, and detailed vitamin and mineral assay.

² Generously supplied by the Corn Products Refining Co., New York.

a *P* value of 0.01 or less were considered significant (*t* equal to, or greater than, approximately 3.0). The standard error of the mean is given with the data.

RESULTS

Similar methionine excretion values were obtained in the first experiment whether the protein ingestion was such that the average intakes were 6–27 mg. of methionine/100 g. body weight/day, as shown in Table I. The intraperitoneal injection of a test supplement of methionine of one or two times the average daily intake of that amino acid on the day the urine was collected, gave small but significant increases in the urinary excretion. Nevertheless, the excretion after the test supplement was not affected by the difference in the levels of the dietary intake of methionine.

TABLE I

Effect of Methionine Intake and Injection on Methionine Excretion

Ten rats, weighing approximately 240 g., were included in each group. The methionine was determined on 24 hr. urine collections of each animal with both intake and excretion values calculated on the basis of 100 g. body weight.

Dietary protein	Period on diet	Methionine		
		Intake per day	Injected last day	Excreted last day ^a
<i>per cent</i>	<i>days</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
5	22	6	0	2.0 ± 0.1
25	22	27	0	2.2 ± 0.1
5	33	6	30	4.6 ± 0.3
25	33	25	30	5.0 ± 0.3
5	36	6	60	12.5 ± 0.6
25	36	25	60	11.8 ± 0.7
Stock	30	60	0	4.6 ± 0.2

^a Standard error of mean is included.

The second experiment was designed to determine whether the greater excretion of methionine after the stock diet was associated with the ingestion of more methionine or less fat. The stock diet contained approximately 5% fat as compared with 40% in the synthetic diet. Similar methionine excretions were obtained on the third day from the groups fed the stock and the synthetic 5% fat diets. In Table II it may be seen that the other groups, with 15% or more fat in the diet, ex-

TABLE II

Methionine Excretion after Varied Protein and Fat Intakes

Urinary methionine was determined on collections made on the third day on these diets. Each group included 8 rats weighing about 350 g. Methionine intake was calculated from daily food intake, and both intake and excretion values based on amounts per 100 g. body weight.

Diet			Methionine	
Protein	Fat	Fat Intake	Intake	Excretion last day
<i>per cent</i>		<i>g.</i>	<i>mg.</i>	<i>mg.</i>
Stock diet		0.34	60	5.2 ± 0.5
25	5	0.29	45	4.9 ± 0.4
25	15	0.79	41	3.6 ± 0.1
5	15	0.92	7	3.6 ± 0.3
25	40	2.39	46	3.8 ± 0.4
50	40	1.52	59	3.5 ± 0.5
Fasted			0	2.3 ± 0.2

creted less (*t* values 2.5–4.7) but similar amounts of methionine whether the diets contained 5 or 50% protein, with actual dietary intakes ranging from 7 to 59 mg. of methionine/100 g. body weight /day. Likewise, significantly less methionine was excreted when the animals were fasted for 3 days.

TABLE III

Effect of Methionine or Choline Injection on Urinary Methionine after Food or Fast

Each group included 8 rats averaging 315 g. Urine for methionine determinations was collected on third day of each dietary regime. Choline was injected each of the 3 days, while methionine was given on the third day only. All values are given in amounts/100 g. body weight/day and are averages of individual analyses.

Dietary regime	Methionine intake	Material injected		Methionine excretion	
		Methionine	Choline	Absolute amounts	Excess due to injection
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
Stock	60			5.0 ± 0.4	
Stock	64	50	—	9.1 ± 0.5	4.1
Fasted	0			2.7 ± 0.2	
Fasted	0	50	—	6.4 ± 0.8	3.7
Fasted	0	—	20	4.1 ± 0.7	1.4

In the third study, test supplements of methionine were given fed and fasted animals in order to compare the excretions in normal and deficient states. After correction for the basal excretion, it is evident in Table III that a similar amount of the methionine of the test supplement injected was excreted whether the animals were fed or fasted. Other data not included supported this finding. The daily choline injections during the 3 day fast were found to be associated with an increased amount of methionine in the urine collected on the third day.

It is interesting that the level of excretion after the choline supplement approached that previously found when the animal was fed a low fat diet, since any apparent differences between the 5.0 and 4.1 excretion values are statistically insignificant.

DISCUSSION

A number of investigators (1,2,10) have reported the urinary methionine to be unaffected by the protein of the diet and a similar result was obtained in this study. However, one might have expected the greatly increased need after such an extended period of deficient protein and methionine intake to be associated with a decreased excretion of that amino acid, which did not occur. To the contrary, a decreased excretion was obtained after the higher fat intake and fasting. An increased metabolism of fat is associated with both of these conditions and may involve an increased need of methionine as a lipotropic substance. The fact that similar methionine excretions were obtained after both the 15 and 40% fat diets suggests that other factors may be involved. The inclusion in the diet of a higher content of fat with the depression of the relative amounts of the other components, and hence their intake, might have some effect independent of the fat itself. However, the effect of changes in the protein and vitamin intakes may be ruled out, since the protein percentage was increased and a constant vitamin supplement was given, regardless of the amount of diet ingested. Thus the increased fat metabolism does seem to be involved in some manner. The amount of methionine in the urine does appear, at least in this case, to serve as an indication of its increased need or utilization.

This increased need for methionine in rats does not seem to be indicated by a decreased urinary excretion of a test supplement of the free amino acid. A similar amount of the supplement of one or two times

the daily intake was excreted, even after an increased need over an extended period of deficient protein intake, as evidenced by lack of growth and fatty livers. As opposed to our findings with rats, Homburger (10) has reported a decreased excretion of a supplement by man after low protein diets. Not only did the low protein diets have no effect upon the excretion of the test supplement in rats, but even the excretion of such after a 3 day fast appeared to be similar to that from the fed animals after a like test supplement, as shown in Table III.

After choline supplements of twice the daily requirement (11), the increase in the methionine excreted to a level approaching that excreted on a wide range of protein intakes suggests a rather constant urinary methionine excretion under usual conditions. This increase after the choline might be interpreted as methionine spared by the choline given, and as supporting the suggestion that at least a part of the decreased excretion when fasting was due to its use to meet greater demands for lipotropic or other needs. This is in agreement with the finding of Salmon (12) that the primary deficiency in low casein diets was that of the labile methyl groups which can be supplied by a choline supplement.

SUMMARY

Wide variations in the protein, and hence methionine, intake of the rat over an extended period did not influence the excretion of that amino acid. A decrease in the excretion of urinary methionine did occur after the higher fat intakes or while fasting, and this decrease may be the result of an increased need of methionine to meet its requirements during an accelerated fat metabolism. However, increased needs associated with a 3 day fast or an extended time on a low protein diet do not appear to be reflected in the excretion of a test supplement of the free amino acid in these studies.

The increased excretion of methionine by the fasting animal when given daily supplements of choline, might be interpreted as a sparing of some of the methionine that would otherwise have been used in the formation of choline, a lipotropic agent. The small increase in amounts excreted following a test supplement of the free amino acid equal to double the daily dietary intake indicates that it is largely metabolized, to be used either unchanged or as some degradation product.

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Folic Acid Metabolism Studies. I. Occurrence of Blood Conjugases^{1,2}

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INTRODUCTION

The importance of folic acid (pteroylglutamic acid, vitamin B₉) for various microorganisms and animals, including man, has become well established in the past few years. These findings have been summarized in several recent reviews (1-5).

Microbiological methods for the determination of folic acid have been complicated by several factors, particularly by the occurrence of several compounds that have folic acid activity which varies not only with different compounds but with different test organisms, by the lack of adequate methods for the complete liberation of the vitamin from its conjugates and by the variable occurrence of inhibitors. Partially purified conjugase preparations from chick pancreas (optimum activity at pH 7.0) and from hog kidney (optimum activity at pH 4.5) for the liberation of folic acid have been described (6-10). Conjugases refer to enzymes that liberate microbiologically available folic acid from more complex forms of the vitamin.

In the course of studies on the influence of the dietary intake of folic acid on the concentration of the vitamin in the tissues, particularly in blood, and on the stability of the vitamin (11-14), the occurrence of folic acid conjugase in blood has been demonstrated.⁴ Studies were conducted on the pH optimum for the release of folic acid, measured with *S. faecalis* R as the test organism, on the occurrence of the enzyme in the blood of several animal species, and on the release of folic acid from blood by the use of several enzyme sources measured both with *S. faecalis* R and *L. casei* as the test organisms.

¹ We are indebted to Frances Panzer for valuable technical assistance.

² Supported in part by a grant from Lederle Laboratories Division, American Cyanamid Co.

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⁴ A preliminary report on these findings was presented at a meeting of the American Society for Biological Chemists, Atlantic City, N. J., March, 1948 (15).

Earlier studies had revealed that the amount of folic acid in turkey blood was the same when the blood samples were incubated with takadiastase as the enzyme source regardless of whether the turkeys had received high or low levels of the vitamin in the diet (14). The amount of the apparent free folic acid which was determined without takadiastase treatment did, however, reflect the dietary intake of the vitamin. In subsequent tests to be described, it became evident that not only was takadiastase ineffective in releasing folic acid from folic acid conjugates, but that conjugases occurring in the blood were capable of releasing folic acid from conjugates in the takadiastase as well as from conjugates in the blood.

EXPERIMENTAL AND RESULTS

The collective term, folic acid, will be used throughout and includes those compounds that are active in promoting growth of the test organisms with the test conditions used. In the experiments to be described, *S. faecalis* R was used as the test organism (16) and some of the later experiments were also conducted with *L. casei* (16) and synthetic pteroylglutamic acid was used as the standard.

TABLE I

Effect of pH of Incubation on the Activity of Folic Acid Conjugase in Blood
(All values expressed as $m\gamma/ml.$)

Source of blood	Treatment of the sample ^a					pH 7.0
	None	pH 4.5	pH 6.0	pH 7.0	pH 8.0	Chick pancreas
Turkey						
whole blood	23.8	26.6	60.2	62.2	86.8	240
Plasma	9.1	7.5	70.2	73.8	57.3	160
Rabbit						
whole blood	4.6	4.4	41.1	27.5	19.8	194

^a Samples incubated for 16-18 hr. at 37°C. Each figure is the average of 4-12 determinations.

In the first experiments the influence of the pH of incubation on the release of folic acid from blood by blood conjugases was determined and the values for folic acid compared with those obtained before incubation (Table I). The latter figures are referred to as apparent "free" folic acid and are indicated for samples receiving no treatment. Oxalated blood was diluted with appropriate quantities of water and assayed directly or when it was necessary to preserve the samples for a period of time prior to assay, the samples were autoclaved and filtered prior to assay. The latter treatment was used in most cases.

TABLE II

Release of Folic Acid from Blood or the Heptaglutamate by the Use of Blood Conjugases or Chick Pancreas as the Enzyme Source
(mγ/ml.)

Sample used	No treatment	Autoclaved	Incubated at pH 7.0	Incubated with chick pancreas pH 7.0
Turkey whole blood	25.7	20.9 ^a	86	354
Plasma	18.9	—	77	153
Rabbit Whole blood	4.5	2.5	24.8	132
Whole blood + conjugate ^b				
Turkey	13.1		105	
Rabbit	2.5		31.9	
Cattle	2.5		16.9	
Plasma	2.9		58.8	
Serum	2.5		40.0	
Human	<2		45.8	

^a Other tests were conducted with blood autoclaved and incubated for 16–18 hr. and the values were similar to those obtained when the same samples were autoclaved only (21.8 as compared to 20.9).

^b Pteroylheptaglutamic acid equivalent to 500 mγ of pteroylglutamic acid per flask.

TABLE III

Effect of Incubating Blood at pH 4.5 and 7.0 on the Release of Folic acid in the Presence or Absence of Pteroylheptaglutamic Acid
(mγ/ml. blood)

pH	4.5		7.0		No treatment ^b
Conjugate added ^a	—	+	—	+	
Series					
I		140		127	8.7
II		65.4		25.4	5.5
III	19.9	121	30.0	119	17.1

^a Equivalent to 500 mγ of pteroylglutamic acid/flask.

^b Not incubated, diluted for assay of the free folic acid content (see text).

When the blood samples were autoclaved and filtered prior to the determination of "free" folic acid (Table II) a slight reduction in the values was obtained, suggesting that some stimulating factors were removed by this procedure, as the stability of pteroylglutamic acid was unaffected by these manipulations. The autoclaving procedure was used in subsequent tests also because, if diluted blood were merely refrigerated for 24 hours prior to assay, occasionally an increase in the values was obtained, presumably due to a slow release of the vitamin by conjugase action. The relatively low concentration of the free folic acid observed in mammalian blood as compared to avian blood is in accord with previous findings (13).

The incubation studies were carried out by using 0.75 or 1 ml. of whole blood or plasma with 5 ml. of 0.2 *M* phosphate buffer, sufficient water to make a total volume of 20 ml., and the final pH was adjusted to 6, 7, 8, or 9. Similar aliquots of these blood samples were incubated at pH 4.5 with an acetate buffer (1 ml. of 2.5 *M* sodium acetate and HCl). After incubation at 37°C. for 16–18 hr., all samples were adjusted to pH 7.0, autoclaved for 10 min., diluted to an appropriate volume, filtered, and assayed for folic acid. A summary of a series of these tests on the effect of pH of incubation on the release of folic acid from turkey and rabbit blood is shown in Table I. These studies showed that the optimum pH for the release of the vitamin was from 6 to 8 and not at 4.5. The release of the vitamin at pH 9.0 was also very low. It is of interest that the pH optimum for the conjugase preparation from chick or turkey pancreas is also pH 7.0, while that from hog kidney is pH 4.5 (6–10).

A further release of the vitamin was affected by incubating the blood samples with an additional source of the conjugase, namely, chick pancreas.⁵ Five mg. of an acetone-dried preparation of chick pancreas were incubated with phosphate buffer at pH 7.0. Samples of the enzyme preparation were incubated in each series and corrections for the vitamin content made in all cases. The values for the enzyme blank ranged from 3 to 10 mγ/mg. of product.

These experiments were useful in indicating that conjugases were present in both avian and mammalian blood and that the release of folic acid was markedly influenced by the pH of incubation. After crystalline B₁₂ conjugate (pteroylheptaglutamic acid) became available,⁶ it was possible to extend these studies and to show a further increase when the conjugate was incubated with blood, thereby relying on the blood only as a source of the enzyme. The occurrence of the enzyme could therefore be more adequately demonstrated and was shown to be present in the blood of several species, including man (Table II). Four to 8 tests were conducted with each source of conjugase.

The high activity of the blood conjugases in releasing folic acid was further substantiated in other tests. For example, when chick pancreas was incubated with blood low in conjugate content, the values obtained averaged 29.4 mγ/ml., while, when a source of conjugate was added but no chick pancreas, the values averaged 135 mγ/ml. of blood (see also Table IV). These and subsequent incubation studies either with blood or chick pancreas as the enzyme source were conducted at pH 7.0 for 16–18 hr.

⁵ Dr. L. R. Richardson generously supplied this preparation.

⁶ We are indebted to Dr. R. A. Brown, Parke, Davis and Company for this compound.

TABLE IV

Effect of Sample Treatment and Enzyme Source on the Amount of Folic Acid Found After Analysis with Either S. faecalis R or L. casei as the Test Organism (mγ/ml.)

Sample treatment	None		Incubated with pteroylheptaglutamic acid		Incubated with chick pancreas	
	<i>S. faecalis</i>	<i>L. casei</i>	<i>S. faecalis</i>	<i>L. casei</i>	<i>S. faecalis</i>	<i>L. casei</i>
Whole blood ^a						
Turkey						
Exp. 1	9.3	14.7	108	131	38.6	—
Exp. 2	11.8	16.5	113	275	26.1	53.5
Human	<2	<2	29.6	128		

^a Six or more samples included in each experiment.

at 37°C. Extending the length of incubation time to 40–42 hr. did not increase the amount liberated. Purification studies with the enzyme have not been included in this work; however, plasma and serum were found to be good sources of conjugase.

In comparisons to test the efficacy of takadiastase as a source of conjugase, turkey blood that had been autoclaved previously to inactivate the conjugases and blood that was not autoclaved, were incubated with 50 mg. of takadiastase at pH 4.5. No liberation of folic acid was observed when the blood was autoclaved, while the "apparent" liberation with unautoclaved blood as observed previously was appreciable. The amount found after treating unautoclaved blood with takadiastase ranged up to an apparent concentration of 400 mγ/ml. of blood; values which were often much higher than those obtained with chick pancreas treatment of the same samples. Similar findings were obtained for rabbit blood. Further evidence for the complications associated with the use of takadiastase is that when takadiastase was autoclaved and then incubated either with blood (pH 4.5) or chick pancreas (pH 7.0) as a source of conjugase, a marked release of folic acid occurred. These data demonstrated, therefore, that not only was takadiastase an ineffective source of conjugase, but contained conjugates of folic acid which were cleaved, in part at least, by either blood or chick pancreas conjugases. Further, folic acid was not released from pteroylheptaglutamic acid by takadiastase but was by blood or chick pancreas enzymes. The ineffectiveness of takadiastase observed in releasing folic acid from the conjugates is in agreement with other reports (8,17,18). The release of folic acid by chick pancreas was reduced somewhat when the blood was autoclaved, which may be due in part to reducing the total enzyme available since the blood enzymes have been inactivated.

These experiments also indicated that if conjugases in blood were capable of releasing folic acid from takadiastase, that the effect of the pH of incubation on the release of the vitamin was different in this case than when blood was used as the substrate. This was clear, since little or no release of folic acid occurred when blood only was incubated at pH 4.5 (Table I), while, when takadiastase was added to blood,

marked increases in the liberation of folic acid occurred. To obtain further information on this point, blood was incubated at pH 4.5 or 7.0, with or without the addition of pteroylheptaglutamic acid as the substrate. In 3 different series of tests, it was noted that at pH 4.5 and 7.0 effective, and essentially equal, releases of folic acid were obtained when pteroylheptaglutamic acid was added to blood and incubated (Table III). Control tests showed that, when blood itself was incubated, a greater release of folic acid occurred at pH 7.0 than at 4.5 (see also Table I). The apparent differences in results on the pH of incubation may be explained on the basis that pteroylheptaglutamic acid and the conjugates of takadiastase are different conjugates than those that occur in the greatest amounts in blood. Also, if several conjugases with different pH optimums and, if several conjugates are present or added, the results obtained represent the overall effects of these factors and the specific influence of each cannot be readily evaluated. Purification studies on the blood enzymes and of the substrates present in blood will aid in clarifying these observations. A recent report (19) showed that a similar phenomenon occurred when rat liver homogenates were incubated, either with or without the addition of the heptaglutamate. Specifically, it was shown that little release of folic acid occurred when liver homogenates were incubated at pH 4.5, but large amounts were released when the heptaglutamate was added prior to incubation at pH 4.5. Effective release of folic acid occurred, however, when liver homogenates were incubated at pH 7.0.

These findings indicate the need for caution in the use of enzyme preparations for the liberation of vitamins. In the case of folic acid, the apparent increases noted when fresh animal tissues, including blood, were incubated with takadiastase (13, 14, 20-23) may be attributable, at least in part, to the release of folic acid from takadiastase by the conjugases present in the fresh tissues. Further, the apparent losses of folic acid attributed to processing of animal and plant foods (12, 23) may be questioned, since the naturally occurring enzymes in the fresh tissues would be inactivated by the processing procedures and, therefore, be incapable of releasing the vitamin from the takadiastase. The correction for the enzyme blank for takadiastase in the usual procedures, therefore, is not always valid, since variable release of the vitamin occurs with the sample tested, depending on the conjugase activity of the sample. Where comparative tests have been made on samples of presumably similar conjugase activities, relative results obtained with takadiastase may be valid. Further studies are needed to reliably evaluate the stability of the vitamin in the processing of natural materials.

In other studies, comparative values for "free" folic acid in blood, and for incubation experiments either with added pteroylheptaglutamic acid or with a chick pancreas preparation with *S. faecalis* R and

L. casei as the test organisms, have been obtained. These results are shown in Table IV. The values for free folic acid in turkey blood are higher as measured by *L. casei* than as measured by *S. faecalis* R. Results tabulated for the amount of folic acid measured after incubating blood samples in the presence of pteroylheptaglutamic acid show even a greater difference as determined with the two organisms, particularly for the studies with human blood. These studies afford an opportunity, therefore, to evaluate the partial degradation products of enzyme action. Thus, when higher values are obtained with *L. casei*, compounds that are active for this organism but essentially inactive for *S. faecalis* R, such as pteroyltriglutamic acid (2, 4), presumably have been formed from the microbiologically inactive pteroylheptaglutamic acid. Further, if lower values are obtained with *L. casei*, one would have evidence suggesting the formation of pteric acid or other compounds (4, 24-26) essentially inactive for *L. casei* but active for *S. faecalis* R. The effect of dietary treatment with respect to folic acid on the concentration of the vitamin in tissues as determined with both test organisms would also be of value in indicating the nature of the folic acid derivatives present.

The possible contributions by thymine and related compounds present in blood and other tissues to the folic acid values as determined cannot be evaluated. The relative values obtained for the different experimental treatments appear to be reasonably specific for pteroylglutamic acid.

It will be noted that, with the use of the blood conjugases or chick pancreas preparations, that not all of the potential folic acid was liberated from pteroylheptaglutamic acid. The liberation obtained in several individual experiments approximates 30% with the conditions used but has not exceeded 50%. For complete liberation, it would appear that preliminary treatment of samples with a proteolytic enzyme low in conjugate content and subsequent treatment with a conjugase preparation such as that from chick pancreas, hog kidney, blood or rat liver offers considerable promise.

DISCUSSION

The occurrence of enzymes in blood and also in other tissues, particularly those enzymes that show activity at pH 7.0, is of significance with respect to their action either in the utilization of conjugates ob-

tained from the diet or by systemic administration. This may well be the explanation for the effective utilization of conjugates by folic acid-deficient chicks and poult (27-29) and by normal human subjects (1, 4, 30-32). Whether the relative inability of some pernicious anemia patients to utilize pteroylheptaglutamic acid can be based only on the presence of inhibitors in the supplements or, at least in part, on the possible limited supply or lack of conjugases in the system, remains to be studied. Further, the conjugase activity of the tissues of mice with spontaneous breast tumors that regress when pteroyltriglutamic acid is supplied (33) may be of importance. Studies conducted with turkeys maintained on purified diets deficient in folic acid indicate that the blood conjugase activity is at least as high as, or higher than, the activity of enzymes from turkeys fed adequate quantities of the vitamin (29). This apparent higher concentration of the enzyme may be an adaptation to the associated lowering of the available substrate.

SUMMARY

The occurrence of folic acid conjugase in blood from several animal species including man, has been demonstrated. The optimum pH of activity determined with turkey and rabbit blood ranges from 6 to 8 rather than in the region of 4.5. Blood conjugases release folic acid from pteroylheptaglutamic acid and the values obtained are considerably higher when measured with *L. casei* as the test organism than when measured with *S. faecalis* R.

Takadiastase has been shown to be ineffective as a source of conjugase and has been shown to contain conjugates which, in the presence of conjugases in the sample, result in abnormally high folic acid values.

A difference in the effect of the pH of incubation on the release of folic acid from blood and from pteroylheptaglutamic acid by blood conjugases was obtained.

The significance of these findings in relation to methods of analysis and utilization of conjugates by the animal is discussed.

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Folic Acid Metabolism Studies. II. Effect of Dietary Intake on the Concentration of Free and Combined Vitamin in the Blood of the Turkey¹

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INTRODUCTION

It has been shown that the blood level of free folic acid was reduced when turkeys were fed diets low in the vitamin as compared to the blood levels when 2 mg. of pteroylglutamic acid was fed in addition to the basal ration (1). These and subsequent studies (2) also revealed that the level of free folic acid was higher in avian blood than in mammalian blood and that conjugases in turkey blood were capable of releasing the vitamin when the blood was incubated either with or without an additional source of folic acid conjugates (2). When day-old turkeys are fed folic acid-deficient diets, a mild anemia, cervical paralysis, lack of growth and death occurs (1, 3-5).

It seemed desirable in the present work to obtain additional data on the influence of the folic acid intake on the amount of free folic acid in blood and the amount measured after enzymatic treatment of the blood. These measurements were made at intervals during the course of the production of the deficiency in turkeys 2-3 months of age and determinations were also made of the blood concentrations of the vitamin following supplementation with pteroylglutamic acid or pteroyltriglutamic acid. These findings have been obtained in addition to data on the rate of growth, feather development and red blood cell volume.

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EXPERIMENTAL AND RESULTS

Young broad-breasted Bronze poulters that had received a standard ration were used in these studies. In the first experiment poulters with an initial weight of 1.6 kg. were used and were approximately 3 months of age. Eight were fed a purified basal ration low in folic acid and four were fed 2 mg. of pteroylglutamic acid/kg. in addition to the basal ration. The detailed composition of the basal ration has been described previously (1). The major components of the ration used were (in per cent) crude casein 25, gelatin 10, corn oil 4.25, vit. A and D conc. 0.75, mineral mixture 5, cystine 0.2, and starch to 100. Adequate quantities of vitamins with the exception of folic acid were provided. The birds were fed these diets for a period of 9.5 weeks. At this time, 3 of the birds fed the deficient diet were fed 2 mg. of pteroylglutamic acid/kg. of ration, and 3 were fed pteroyltriglutamic acid equivalent to 2 mg. of pteroylglutamic acid/kg. for a 2 week period.

The birds fed the deficient diet gained at a slower rate, showed poor feather growth and leg weakness, and became anemic. The average rate of gain per week for the 9.5 week period was 360 g. for the supplemented group and 281 g. for the deficient group. The deficient group was still gaining slowly during the latter phases of the experiment.

TABLE I
Effect of Folic Acid Intake on the Red Blood Cell Volume and Free Folic Acid in Plasma

Dietary treatment	Red blood cell volume		Free folic acid in plasma	
	-F. A.	+F. A.	-F. A.	+F. A.
<i>weeks on experiment</i>	<i>per cent</i>	<i>per cent</i>	<i>mg/ml.</i>	<i>mg/ml.</i>
4	30.7	37.2	3.4	19.0
6	33.1	39.3		
8	28.0	40.1	1.2	18.3
9.5				
	+F. A.		+F. A. ^a	
10	38.7	42.1	Group A 29.8	Group B 24.8
11.5	39.2		33.7	41.0
				35.2

^a Group A received pteroylglutamic acid and Group B pteroyltriglutamic acid.

Red blood cell volumes were determined after the birds had been on experiment for 4, 6, and 8 weeks, and 2 weeks after supplementation of the deficient group. A striking difference in the hematocrit values was obtained, and after 8 weeks on experiment the values averaged 28.0% for the deficient group and 40.1% for the supplemented group (Table I). Six of the 8 birds in the deficient group had hematocrits below 31%. One of the values obtained was only 11.8%, and following supplementation the hematocrit level increased to 26.7% in 4 days, and to 33.3% in 14 days. The average for the group of 6 birds supplemented with either pteroylglutamic acid or pteroyltriglutamic acid two weeks after supplementation was 38.4%.

In the second experiment 8 poults, 2 months of age and with an initial weight of 900 g., were fed the deficient diet and 5 comparable poults were fed the supplemented diet. They were continued on this regimen for 7.5 weeks. Three of the 8 birds fed the deficient diet died during the 4th-6th week of the experiment. During the last few days before death, the birds became very weak and were unable to walk. One of the other birds, which was unable to walk, was injected intraperitoneally with 500 γ of pteroylglutamic acid and recovered. All birds fed the deficient diet showed a poor feather condition. The control group gained at a rate of 336 g./wk. for the first 6 weeks, and the surviving birds in the deficient group gained an average of 265 g./wk. for a similar period. A small reduction in the red cell volume was produced in this series when the deficient diet was fed.

After 7.5 weeks on experiment, 2 of the remaining birds were supplemented with 2 mg. of pteroylglutamic acid/kg. of ration, and 3 were fed an equivalent amount of pteroyltriglutamic acid (3.16 mg.). The food was withheld from the poults for 1-2 hr. and blood was then taken by wing vein. Samples of whole blood or plasma were taken at regular intervals throughout the experiments and analyzed for their free folic acid contents (2). Other aliquots of the samples were taken for determinations of combined forms of the vitamin. These determinations were obtained after incubating the samples with 5 mg. of a chick pancreas preparation for 16-18 hr. at 37°C. The values obtained without treatment of the sample have been referred to as "apparent free folic acid" (2,6) and those after enzyme treatment include measurements of both free folic acid and that liberated from conjugates in the blood. The latter compounds are microbiologically inactive prior to treatment with chick pancreas or some other source of conjugase. The values obtained with the chick pancreas treatment reflect the relative amounts of the combined forms of folic acid in the blood samples for the 2 groups within each series of tests.

The diluted samples of whole blood and the chick pancreas digests were autoclaved for 10 minutes to inactivate the enzymes, filtered and diluted as needed for assay. Folic acid was determined with the use of *S. faecalis* R as the test organism (7) and in a few tests conducted in the second experiment, corresponding analyses were obtained with *L. casei* (7).

The results obtained in the first experiment are summarized in Fig. 1 (Charts A and C), and for the second experiment in Fig. 1 (Charts B and D). Data were also obtained for the free folic acid content of plasma in the first experiment and are presented in Table I. All values were obtained with *S. faecalis* R as the test organism. The sequence of experimentation during the course of the deficiency and the results after supplementation of the deficient poults are also presented. It can readily be seen that the level of the vitamin in the blood does markedly reflect the dietary intake of the vitamin. This was observed after only 2-4 weeks on experiment and was particularly evident in the values obtained for the free folic acid content. The differences were most marked in the values for plasma. The relative differences in combined

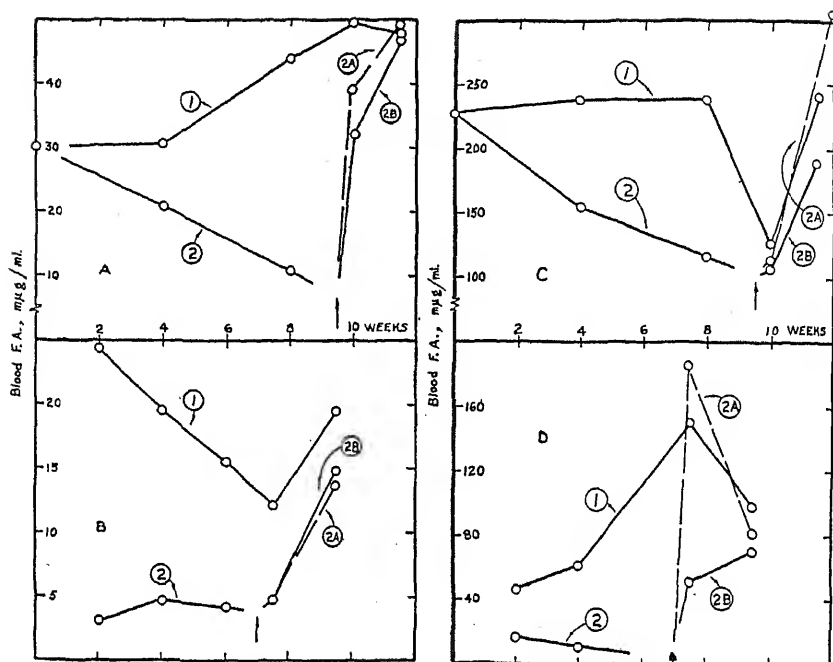


FIG. 1. Effect of ingesting folic acid-deficient or supplemented diets on the amount of folic acid in the blood. Charts A and B—Results obtained in Expts. 1 and 2, respectively, on the free folic acid level of the blood, and Charts C and D, the respective levels of folic acid determined after treatment of the blood with a chick pancreas preparation (see text). Curve 1 was obtained for the groups fed the supplemented diet, and Curve 2 for the groups fed the deficient diet. Curve 2A was obtained when the deficient birds were fed 3.16 mg. pteroyltriglutamic acid/kg of ration, and Curve 2B was obtained for the deficient birds fed 2.0 mg. of pteroyltriglutamic acid/kg of ration. The arrow indicates the time of supplementation.

folic acid determined after chick pancreas treatment also reflect the effect of dietary treatment. Considerable variation was observed in the values from one series of tests to the next, particularly in the amounts liberated by chick pancreas treatment. The role of the different factors involved, such as the degree of liberation and concentration of conjugates present, cannot be readily evaluated. The relative values obtained in each test for the deficient and supplemented groups were obtained under the same conditions of analysis, however.

When either pteroylglutamic acid or pteroyltriglutamic acid was given to the deficient birds, a rise in the folic acid level was clearly demonstrated and the amount present appeared to be essentially at a normal level within 2 weeks after supplementation. The triglutamate derivative appeared to be equally as effective as pteroylglutamic acid in elevating the level of the free vitamin in the blood. These results are in agreement with other reports that the triglutamate is as effective as pteroylglutamic acid in promoting growth and hemoglobin production in chicks (8, 9). The elevation in the values obtained after chick pancreas treatment when the triglutamate was fed as compared to pteroylglutamic acid suggests the presence of greater amounts of conjugates in blood when the former compound was fed. The exact nature of these conjugates cannot be stated.

A few comparative tests were also conducted with *L. casei* as the test organism. The results obtained with this organism were also in accord with the dietary treatment. The values obtained after chick pancreas treatment of the samples, in particular, were higher than those obtained with *S. faecalis* R. This indicated that conjugates more active for *L. casei* than for *S. faecalis* R were released. In one series, for example, the values for the deficient and supplemented groups were 9.3 and 59.8 m γ /ml., respectively, after chick pancreas treatment as determined with *S. faecalis* R, and 34.0 and 82.1, respectively, as determined with *L. casei*.

These techniques should be valuable for obtaining further information on the folic acid content, both free and combined, of tissues from animals receiving various levels of the vitamin. Further, by the use of both test organisms, some interpretations can be made of the type and concentration of folic acid derivatives that are present. The turkey is an excellent test animal for these studies in that sufficient blood can be obtained for a number of tests during the course of the dietary treatments and it responds rapidly to the ingestion of diets deficient in folic acid.

SUMMARY

The influence of the dietary intake of folic acid on the level of free and combined vitamin in the blood of the turkey has been determined. The blood concentrations were determined in 2 experiments at regular intervals, when turkeys 2-3 months of age were fed a purified diet con-

taining no added folic acid, or the same diet plus 2.0 mg. of pteroylglutamic acid per kg. of ration. The poult fed the deficient diet developed a mild to severe anemia, poor feather condition, weakened legs and unthrifty appearance, as well as a marked reduction in the blood folic acid levels. When pteroylglutamic acid or pteroyltriglutamic acid was fed to the deficient poult, a prompt recovery occurred. The results indicate that both compounds were equally active on a molar basis, and that the blood levels of the vitamin were essentially normal 2 weeks after supplementation was initiated.

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Amino Acid Analyses of the Nondiffusible Fraction of Enzymotic Protein Digests and Human Urine

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INTRODUCTION

Although the results of amino acid analyses of acid hydrolyzates of proteins obtained by the application of chemical and microbiological procedures are in fair accord, considerable differences have been reported to exist between the results of chemical and microbiological analyses of biological fluids and enzymotic digest of proteins. Some causes for these differences would appear to be: (a) the abnormal growth response of microorganisms to bound amino acids, (b) the inability of chemical methods to differentiate between amino acids and their metabolic derivatives, and (c) the lack of specificity of particular chemical and microbiological procedures. Since clarification of these differences is necessary not only for the authentication of chemical amino acid methods which have been previously described for the urine but also for the evaluation of the metabolic role of urinary substances which are chemically similar, but microbiologically dissimilar, to the amino acids, we have concerned ourselves for some time with the resolution of the sources of these discrepancies. Our first efforts in this direction, which are reported here, were aimed at (a) assessment of the capacity of chemical methods to measure amino acids in peptide form, and (b) the determination of bound amino acids in the urine. The ability of the chemical methods to measure peptide-bound amino acids was tested by analysis of a chemically defined undialyzable component of enzymotic protein digests before and after acid hydrolysis. In this manner it was found that the chemical techniques for methionine (1), arginine(2), histidine (3), tyrosine (4), and phenylalanine (5), yield

quantitative measurements of these amino acids in polypeptide linkages, whereas the β -hydroxy- α -amino-N (6) procedure appears to measure only a moiety of the bound hydroxyamino acids. The results of the cystine analysis indicate that the method of Sullivan (7), as pointed out by the author, measures only free cystine. The quantitative measurement of bound tryptophan by our method (8) could not be assayed in this fashion because of the absence of tryptophan in the undialyzable residue of protein digests, but was demonstrated by analysis of native serum and milk proteins.

These findings with the nondiffusible casein digest fraction indicated that the total bound amino acids of the urine could not be determined as the difference between acid-hydrolyzed and unhydrolyzed urine specimens as we and others had previously thought, and suggested that the bound amino acids of the urine should be differentiated into two fractions, namely, diffusible and nondiffusible. The results of chemical analysis of the nondiffusible N fraction isolated from 20 l. of human urine, which constitutes only a moiety of the urinary amino acids, which would not seem to be readily measurable by microbiological means, disclosed this component to contain significant amounts of arginine, histidine, cystine, methionine, tyrosine, phenylalanine, and tryptophan.

EXPERIMENTAL

Preparation of Nondialyzable Peptides from Enzymotic Casein Digests

Fifty g. of casein (Sheffield, 14.6% N corrected for ash and moisture content) were suspended in 250 cc. of water; then 5 g. of Na_2CO_3 , 0.5 g. of NaF and 2 g. of pancreatin (Takamine) were added in succession with stirring and the mixture stored at 37°C. for 6 hr. after the volume had been adjusted to 500 cc. The enzymic reaction was stopped by acidifying the mixture to pH 3 with 6 N HCl. This solution was transferred to 2 cm. cellophane tubing (Eimer & Amend No. 8-667) and dialyzed overnight against running tap water in the rotary dialyzer shown in Fig. 1. After dialysis, the fraction (biuret positive) which had separated from solution was filtered off and discarded. The filtrate, which was found to give negative Exton's and trichloroacetic acid tests, was concentrated *in vacuo* to a heavy syrup. This concentrate was transferred to a 50 cc. centrifuge tube with the aid of 25 cc. of water, and a white amorphous product was obtained on slow addition of 15 cc. of acetone. The yield of this product was more than doubled by refrigerating the mixture at 4°C. for 48 hr. The product was washed twice by centrifugation with 30 cc. portions of cold acetone and dried over calcium chloride in a desiccator. The nitrogen content of this preparation was not significantly altered by two reprecipitations from aqueous acetone as described below. Approximately 2.5 g. of nondiffusible polypeptides were derived from each 50 g. portion of casein processed in this manner.

Preparation of Nondiffusible Nitrogen Fraction of Human Urine

Twenty-four hr. urine specimens collected in brown bottles containing 20 cc. of 15% HCl (by volume) and 1 cc. of 10% alcoholic thymol from 12 male and female subjects ranging in age from 2 months to 40 years (a total of 20 l. of urine) which gave negative biuret, trichloroacetic acid and Exton's tests, were dialyzed as will be described below. The dialyzed samples were pooled, concentrated *in vacuo* to a thin syrup, and the non-diffusible components separated from solution by the addition of acetone to the point of incipient precipitation. This crude product attained constant N content after the second precipitation from aqueous acetone.

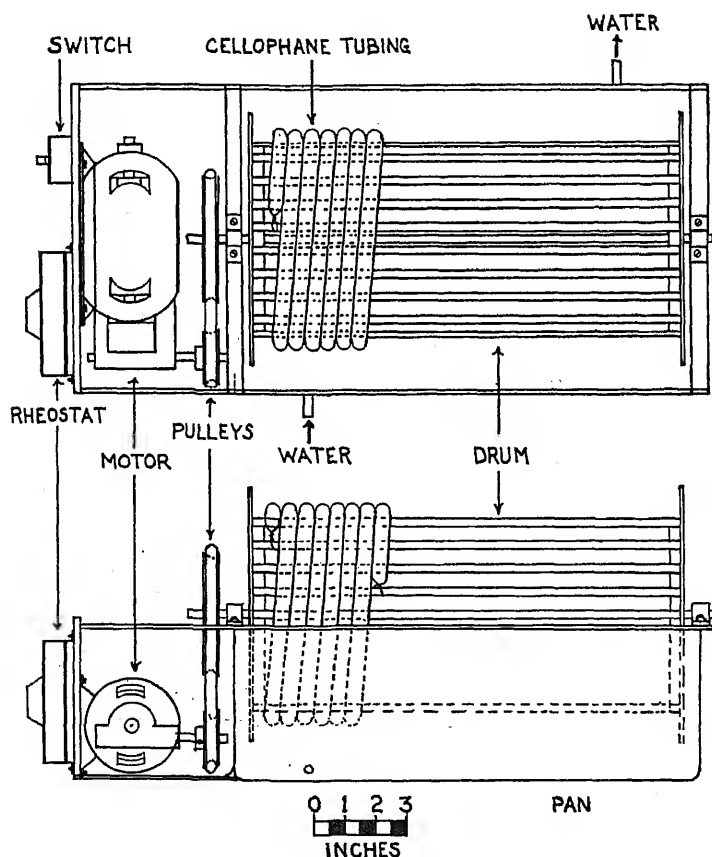


FIG. 1. Drawing of rotary dialyzer used for the preparation of the nondiffusible nitrogen component of enzymic casein digest and human urine.

Dialysis Procedure. The nondiffusible fractions of enzymic casein digests and human urines needed for this investigation were prepared in the rotary type dialyzer shown in Fig. 1. In operation, the required length of 2 cm. cellophane tubing (one inch allowed/7 cc. of solution) is coiled loosely about the drum, and the sample transferred before filling the pan with water. The ends of the filled tubing were securely tied to the crosspieces of the drum and the motor turned on after the water reached the overflow level. The rate of water flow was maintained at approximately 20 gal./hr. The apparatus as shown has a maximum capacity of 2 l., but optimal dialysis rate was attained when only 1 liter of sample was employed. Test runs with 1 liter of urine or 500 cc. of casein digest sample disclosed that constant amino N: total N ratios were attained in both instances with 6 hr. dialysis (Fig. 2). In practice, however, it was found more convenient to continue the dialysis overnight.

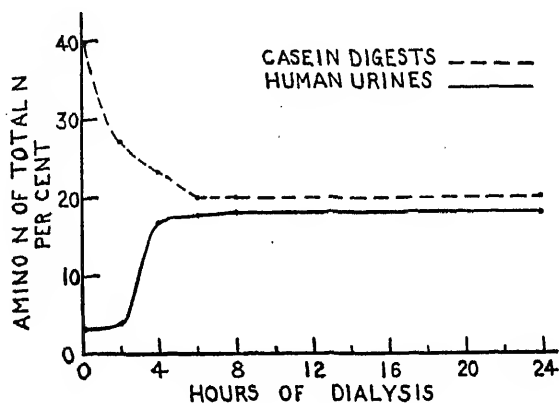


FIG. 2. Rate of dialysis of enzymic casein digests and human urine.

Analyses of Nondialyzable Peptides of Casein Digests

Three 50 g. samples of casein were submitted to enzymic hydrolysis and dialysis as described. The accord (Table I) in specific rotations, ash, moisture, and nitrogen content, indicate that this peptide fraction can be reproducibly prepared in this manner.

TABLE I
Characteristics of the Nondiffusible Component of Enzymatic Casein Digest

Enzymotic casein digest	Yield	$[\alpha]_D^{25}$ of 1.0% solution in water	Ash content	Moisture content	Corrected nitrogen content	Amino N of total N
	g.		per cent	per cent	per cent	per cent
1	2.42	-51.8	16.8	4.5	13.2	19.2
2	2.44	-50.7	17.0	4.5	13.8	19.6
3	2.60	-51.3	17.1	4.4	13.8	19.2

After these preliminary analyses the 3 preparations were combined and aliquots of the composite submitted to chemical and microbiological amino acid analysis before and after the usual 6 N HCl hydrolysis. These results are collected in Table II. From the agreement of the arginine, histidine, methionine, tyrosine, and phenylalanine values of the whole and hydrolyzed peptide fractions, it must be concluded that the chemical methods employed are capable of measuring these amino acids in either the free or bound form. The absence of tryptophan in these casein fractions was demonstrated by the negative tests obtained by the chemical procedures of Albanese and Frankston (8) and Shaw-McFarlane (10), and the microbiological method of Greene and Black (11). The possibility that the negative tests given by the Albanese-Frankston technique might be due to the inability of this procedure to measure bound

TABLE II
*Amino Acid Composition of the Nondiffusible Fractions of
Enzymatic Casein Digests and Human Urine*
(Amino Acid N Distribution in Per Cent of Total N)

Source	Casein digests		Human urine	
	Before	After	Before ^a	After
Acid hydrolysis				
Amino N (9)	23.2	80.1	18.0	61.4
Arginine (2)	3.99	3.93	8.64	8.56
Histidine (3)	5.62	5.66	5.27	5.30
Cystine (7)	0	1.26	0.0	5.04
Methionine (1)	1.17	1.22	4.25	4.38
Tyrosine (4)	0.48	0.51	0.84	0.89
Phenylalanine (5)	3.53	3.50	4.40	4.40
Tryptophan (8)	0.0	—	12.65	—
β -Hydroxy- α -amino-N (6)	1.11	15.7	0.0	0.0

^a Amino acids in nondiffusible fraction calculated in mg./l. of urine: Arginine, 13.1; histidine, 6.7; cystine, 15.2; methionine, 16.2; tyrosine, 3.9; phenylalanine, 18.0; tryptophan, 32.8.

tryptophan was confuted by the fact that this technique has been employed by us for the determination of tryptophan in whole proteins. Thus, we have found that isolated whole human serum proteins and whole cow's milk proteins contain, respectively, 2.2 and 1.4% of tryptophan; values which are in fair accord with those available in the literature (12). It is apparent from the difference in cystine values of whole and hydrolyzed peptides that the procedure of Sullivan, as previously noted, does not measure peptide-bound cystine. The determination of total β -hydroxy- α -amino-N by the periodic acid technique indicates that less than 10% of the hydroxyamino acids are so disposed in the polypeptide molecules as to make their measurement possible by this reaction. It is of interest to note that the ash contained in the fractions consisted

principally of calcium and phosphate ions in such proportions as to give 1.1 and 1.0 *M* equivalents, respectively, with the β -hydroxy- α -amino-N present in the acid-hydrolyzed preparation.

Analysis of Nondiffusible Urinary Nitrogen

Twenty l. of urine, which contained 8700 mg. of amino N in all, were processed as previously described and yielded 6.2 g. of crude nondiffusible product which contained 159 mg. of amino N. Constant nitrogen was attained after two precipitations of the crude preparation from 50% aqueous acetone. This substance was white and amorphous in appearance, weighed 5.10 g. and contained 4.74% moisture, 2.28% ash, and 13.68% N by Kjeldahl. The opalescence of a 1% solution of this product made the determination of specific rotation impossible.

The results of amino acid analysis of aliquots of the urinary product before and after acid hydrolysis are given in Table II. The most striking characteristic of these analytical results is the high tryptophan value found by the Albanese-Frankston method, which measures the free and bound amino acid. A determination of tryptophan by the Shaw-McFarlane method showed this same material to contain only 4.2% tryptophan N of total N, or approximately one-third that found by our procedure. This discrepancy was not surprising to us, as we had previously noted that, although the Shaw-McFarlane and Albanese-Frankston tests yield similar tryptophan values for alkaline digests of casein and lactalbumin, the Shaw-McFarlane procedure gives values which are about 50% lower than those of the Albanese-Frankston technique for enzymotic digests of casein and lactalbumin. It was observed that, although the microbiological assay of the urines employed in the preparation of this sample gave tryptophan values in the range reported by Schweigert and coworkers (13), solutions of the nondiffusible urinary component which contained chemically demonstrable tryptophan, gave negative microbiological readings. These findings, and the fact that the tryptophan contained in this urinary component alone, is greater than the daily tryptophan adult output (12.6–30.5 mg./day) as measured by microbiological methods (13) and (20–42 mg./day) as measured by the Shaw-McFarlane procedure (14), bring into sharp focus the hazards which confront the analyst when measuring organic components of heterogeneous systems such as urine and other biological fluids.

In an attempt to ascertain the manner in which arginine was bound in the polypeptide fraction, aliquots of the urine preparation were tested with a crude arginase prepared from beef liver as described by Hunter and Dauphinee (15). In this manner it was found that only 35% of the chemically determined arginine could be measured enzymotically. A similar experiment with the nondiffusible N fraction derived from the enzymotic casein digests disclosed that none of the colorimetrically estimated arginine could be detected by the enzyme test. After acid hydrolysis, the arginine values obtained by the enzymotic procedure for both these preparations were in good accord with those listed in Table II. These observations, and the further finding that the polypeptides contained in this urinary component are quantitatively adsorbed on activated permutit columns, indicate that the colorimetric determination of urinary arginine is of greater usefulness than the enzymotic method of Hoberman (16) in the construction of human urinary amino acid patterns.

The absence of β -hydroxy- α -amino-N from this urinary fraction is also to be noted.

DISCUSSION

Inasmuch as the chemical reactions employed for the determination of methionine, arginine, histidine, tyrosine, and phenylalanine are known to occur also with these amino acids as they exist in whole proteins (17), it is not surprising to find that the amino acid composition of the nondiffusible casein digest and urinary polypeptides could be ascertained by direct application of these procedures to the intact specimens. Our present experiments further disclosed that the modified Jolles reaction as embodied in the Albanese-Frankston procedure measures free as well as protein-bound tryptophan.

From the data presented, it appears reasonable to implicate the analytical idiosyncrasies of the chemical and microbiological procedures as a source of some of the discrepancies of the results secured by these two analytical principles. These data however do not account for all the existing differences in the urinary amino acid concentration as measured chemically and microbiologically. On the other hand, it must be recalled that these values represent only the nondiffusible fraction of the bound urinary amino acids. Comparable data on the free amino acids and the diffusible bound amino acid fraction of the urine might be expected to further explain the existing differences.

SUMMARY

It has been found that chemical methods for the determination of arginine, histidine, methionine, tyrosine, phenylalanine, and tryptophan, measure these amino acids quantitatively in free or bound form. The presence of appreciable quantities of these amino acids in the nondiffusible moiety of the urinary N may account for some of the discrepancies existing between the microbiological and chemical measurements of the urinary amino acid excretion of man.

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Protein Utilization of Various Dried Food Yeasts ¹

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INTRODUCTION

The literature on the nutritive value of yeast proteins has been reviewed by Carter and Phillips (1) and recently by von Loeschke (2). Klose and Fevold have apparently used strains of brewers' yeast and *Torula* yeast grown in molasses that were deficient in methionine, which accounts for the poor growth they observed when their yeasts were used as a source of proteins for growth of rats (3). However, there seems to be general agreement that yeast protein is readily digested and absorbed by the rat, and that it is a satisfactory protein for the dog (2).

In 1946, Sure reported that a brewers' yeast, strain K,² when used as the only source of proteins and the vitamin B complex, produced excellent growth, reproduction, and lactation, and that cultured yeasts, strains G, 90, and 300² also proved to be very good sources of proteins and the B vitamins for such physiological functions. It was not possible to make accurate comparisons of relative values of these food yeasts, because 4 generations of animals were obtained on the K yeast and studies on the other yeasts were carried out only through the second generation. However, the indications were, judged from the standpoint of growth, reproduction, and lactation, that the K yeast was the best, followed by G, 300, and 90, respectively. It was also found that the K and G yeasts supplemented the proteins in milled wheat flour and milled corn meal to a remarkable extent (4).

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² Supplied by Anheuser-Busch, St. Louis, Mo.

EXPERIMENTAL

In this investigation a study was made of the biological values of the proteins in yeasts K, G, 90, 200, and 300 by the nitrogen balance method of Mitchell (5). The results are given in Tables I and II. To conserve space, all detailed urinary and fecal data are deleted and only figures for true digestibilities and biological values are submitted, as recently presented by Mitchell (6).

TABLE I
Chemical Composition of Dried Food Yeasts

	Yeast K	Yeast G	Yeast 90	Yeast 200	Yeast 300
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Protein (N×6.25)	49.8	44.8	47.9	45.4	51.8
Moisture	5.7	6.0	6.5	5.9	5.3
Ash	8.0	8.3	8.1	7.6	6.6
Thiamine γ /g.	150	150	270	600	900
Riboflavin γ /g.	40	75	75	75	75
Niacin γ /g.	250	500	500	500	500

TABLE II
Protein Utilization of Various Food Yeasts

Yeast	Number of animals	True digestibility			Biological value ^a			Net utilization ^b		
		Per cent SD ^c	Per cent SD	Per cent SD	Per cent SD	Per cent SD	Per cent SD	Per cent	Per cent	Per cent
Brewers' (K)	8	10	8	5	10	8	5	10	8	5
Cultured (G)	8	—	88 ± 3.6	91 ± 2.2	—	66 ± 7.9	66 ± 8.6	—	58	60
Cultured (90)	12	87 ± 2.8	91 ± 1.2	91 ± 2.6	—	68 ± 3.9	70 ± 6.0	—	58	64
Cultured (200)	10	88 ± 2.1	88 ± 1.9	91 ± 3.6	76 ± 6.1	82 ± 6.5	86 ± 6.0	66	75	79
Cultured (300)	8	—	88 ± 2.1	92 ± 3.4	—	79 ± 5.1	89 ± 8.4	64	70	81
						73 ± 7.0	72 ± 7.9	—	64	66

^a Per cent of absorbed nitrogen retained.

^b The value for the true coefficient of digestibility multiplied by the biological value, divided by 100.

^c Standard deviation, which represents the mean error of a single observation.

The total protein content of the yeasts varied from 44.8 to 51.8%, and the thiamine, expressed as γ /g., varied from 150 to 900 (Table I). With the exception of debittered brewers' yeast K, all the yeasts contained the same amounts of riboflavin and niacin. Yeasts K, G, and 300 were studied at 5 and 8%, and yeasts 90 and 200 at 5, 8, and 10% planes of protein intake.

The composition of the rations was as follows, expressed as percentage: Yeasts to furnish in rations 5, 8, or 10% of protein (N × 6.25); cellu flour, 2; lard, 10; Sure's

salts No. 1 (4), 4; cod liver oil, 1.5; wheat germ oil, 0.5; and the balance, cerelose. In addition, the following components of the vitamin B complex were administered daily to each animal separately from the ration: 50 γ of each of the following: thiamine, riboflavin, pyridoxin, and niacin; 300 γ calcium pantothenate, 9 mg. choline chloride, 6 mg. *p*-aminobenzoic acid; and 2 mg. inositol.

Description of Yeast

Brewers' Yeast, Strain K. This is a pure debittered yeast obtained from brewing. It has been dried at pasteurizing temperature and is nonfermentable.

Cultured Food Yeast, Strain G. This is a pure primary yeast grown in a special hop-free media. The process used for manufacturing strain G as well as strains 90, 200, and 300, is primarily for yeast production, hence, are called primary yeasts. No products, such as beer, alcohol, etc., are recovered. These yeasts, according to the manufacturers, are dried above pasteurizing temperature and are nonfermentable.

Cultured Food Yeasts, Strains 90, 200, and 300. These are pure primary yeasts grown in a hop-free media enriched with extractives of by-products from corn products and malting operations. They are also enriched with varying amounts of thiamine. They are dried above pasteurizing temperature and are nonfermentable.

DISCUSSION

It will be noted from Table II that all the yeasts fed at a 5% protein level have true digestibilities of 91–92% and at an 8% protein intake the digestibilities are about 88%.

While yeasts G, 90, 200, and 300 are all primary yeasts grown in vats on ammonium salts as the source of nitrogen and black-strap molasses as the source of calories, in presence of an abundance of air, strains 90, 200, and 300 differ from strain G and brewers' yeast K, since they had added to the culture media extractives from by-products of corn products and malting operations. This may account for their higher biological values. The large standard deviations for the biological values of all the yeasts is due to individual differences in nitrogen retention and to some extent to differences in digestibilities. However, in spite of these great variations, the biological values of the proteins of yeasts 90 and 200 are definitely the highest. Why yeast 300 has a lower biological value than yeasts 90 and 200 we cannot tell at present but it is possible the high thiamine content of yeast 300 may interfere with its protein utilization. These results do not, however, indicate that the yeasts with the higher protein biological values are the most nutritious from the standpoint of all dietary essentials. On the contrary, previous work indicated that yeasts K and G, fed at higher levels of

protein intake, namely, at 18 and 20% in rations, were superior to strains 90 and 300 for growth, reproduction, and lactation.

The low biological values of 48.8 and 45.3 for the types of food yeasts recently reported by Goyco and Asenjo (7) are probably due to the fact that they used strains of yeast containing incomplete proteins. Their figure of 69.3 for the strain of brewers' yeast is, however, close to our figure of 65.6 for the same type of yeast when fed on the same level of 8% protein.

SUMMARY

Protein utilization was studied in 5 strains of dried food yeasts. On a 5% protein level, the biological values and true digestibilities were respectively as follows: K yeast, 66 and 91; G yeast, 70 and 91; 90 yeast, 86 and 92; 200 yeast, 89 and 91; and 300 yeast, 72 and 92. On an 8% protein level, the biological values and true digestibilities were as follows: K yeast, 66 and 88; G yeast, 68 and 86; 90 yeast, 82 and 91; 200 yeast, 79 and 88; and 300 yeast, 73 and 88. On a 10% protein level, the biological values and true digestibilities were as follows: 90 yeast, 76 and 87; and 200 yeast, 73 and 88.

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Utilization of Non-Sugar Carbon of Molasses by Food Yeasts¹

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INTRODUCTION

In the production of food yeast from natural substrates, the yield of yeast is generally calculated on the basis of sugar supplied or fermented. This basis of calculation is somewhat misleading, because it does not take into account the utilization of other organic compounds present in molasses, sulfite waste liquors, wood hydrolyzates, and other natural materials. An extreme case of such fictitious values is illustrated by the work of Peukert (1). When he grew *Biosyn* I (a mixture of *Oidium lactis*, *Fusarium aqueductum* and other fungi (2)) on slops from the alcoholic fermentation of sulfite waste liquors, the yields of dry yeast, based on sugar supplied, were 100%. Peukert realized that such high yields are impossible on stoichiometric grounds, and attributed the results to the utilization of not only the reducing sugars, but also other soluble decomposition products of wood present in sulfite waste liquor. The ability of certain yeasts to utilize organic compounds other than sugars of natural substrates has been noted by a number of investigators. During World War II, the Germans (3) employed certain yeasts like *Torulopsis utilis*, *Torula pulcherrima*, *Monilia candida*, *Candida arborea*, mixed torula, *Oidium lactis*, etc., that can use residual pentoses, acetic acid, and other organic compounds present in still bottoms from the alcoholic fermentation of sulfite liquor. Collingsworth (4) observed further growth of *Saccharomyces cerevisiae*, even

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after the disappearance of sugar, and attributed this to the utilization of non-sugar carbon compounds present in grain-wort. Kurth (5) and Kurth and Cheldelin (6), using wood sugar stillage, reported approximately 63% yields of *T. utilis* (dry basis) on sugar used, and concluded that part of this yeast growth came from organic acids in the liquor. They observed that 36–60% of the total organic acids and 60% of the volatile acid were used by *T. utilis*.

In view of this situation a more accurate method of calculating the yields of yeasts on natural substrates is to base the calculation on the total carbon utilized. Very little attention has been given to this method of calculation. Sperber (7) made a carbon balance and found that more carbon was recovered as yeast and CO₂ than was present in the sugar of the molasses. He attributed this excess carbon as coming from non-sugar compounds.

In this study, utilization of non-sugar carbon of molasses by food yeasts and factors affecting this utilization, have been investigated.

EXPERIMENTAL

Yeast Strains

Three strains of yeasts, *Saccharomyces cerevisiae* No. 53, *Torulopsis utilis* No. 3, and *Candida arborea* were used. Details regarding the origin and use of these strains by others are given in our previous paper (8).

Fermentation

The procedure for growing the inoculum was the same as described previously (8,9). Two and a half ml. of a suspension that contained about 20–35 mg. of dry yeast were used as seed for 50 ml. of medium in 500 ml. Erlenmeyer flasks.

Three samples of beet molasses from Michigan Sugar Company, Lansing, Mich.; American Crystal Sugar Company, Mason City, Ia.; and Great Western Sugar company, Ovid, Colo.; and one sample of cane molasses, Hawaiian blackstrap from California and Hawaiian Sugar Refining Corporation, Ltd., San Francisco, Calif., were used. The samples of beet molasses were clarified as previously described (8). The Hawaiian molasses was clarified as follows: 100 g. of molasses was diluted to 250 ml. with distilled water and adjusted to pH 8.0 with a suspension of Ca(OH)₂. The material was steamed for 20–30 min. and then allowed to stand in the cold room overnight. The precipitate which had formed was removed by filtration after addition of 15–20 g. of Celite 545 (a filter aid made by Johns-Manville Co.). The filtrate was adjusted to pH 4.0 with H₂SO₄ and again steamed for 20–30 min., cooled and filtered as before. When this solution was mixed with corn steep liquor, a precipitate formed which was removed by centrifugation. The solution was then diluted to the desired concentration.

In some of the experiments, the ash of corn steep liquor was used as a substitute for corn steep liquor. This was prepared as follows: 100 ml. corn steep liquor (clarified by steaming a solution of crude corn steep liquor diluted with water 1:3 for 30 min., cooling and filtering) was dried, carbonized over a free flame, and the ashing completed in a muffle furnace at 600°C. A carbon-free ash was obtained by adding 5 ml. of concentrated HCl and again heating in the muffle furnace. The residue was taken up in water, filtered and diluted to 100 ml.

Unless otherwise mentioned, fermentation media contained processed molasses sufficient to give approximately 1% sugar calculated as glucose. Fermentations were run with 50 ml. of medium in 500 ml. Erlenmeyer flasks at 30°C. For aeration, the cotton plugged flasks were shaken on a rotary shaker describing a circle of 4 in. diam. at a speed of 250 r.p.m. Rotating the flasks at this high speed gives a strong swirling movement to the liquid that aerates it very efficiently. That the aeration was adequate is attested by the high yields obtained. These were approximately equal to those obtained on the same media in 30 l. fermenters when optimal mechanical aeration and agitation were employed. Unless otherwise specified, all fermentations were run on the rotary shaker.

Analytical

Yeast growth was determined from the weight of dry yeast cells (8), reducing sugar by the micro method of Shaffer and Somogyi (10) (reagent No. 50 with 5 g. KI/l.) after acid hydrolysis and calculated as glucose. Total carbon was determined by the wet combustion volumetric method of Friedmann and Kendall (11). The method was tested on a number of compounds, $\text{KHC}_8\text{H}_4\text{O}_4$, CH_3COOH and $\text{C}_2\text{H}_5\text{OH}$, etc., and 98–100% carbon recoveries were obtained. Non-sugar carbon was calculated by subtracting sugar carbon from total carbon and non-sugar, non-alcohol carbon by subtracting sugar carbon and ethyl alcohol carbon from total carbon. Figures for carbon in cells were calculated on the assumption that *T. utilis* contains 45.9% carbon (7). Ethyl alcohol was determined by Johnson's procedure (12).

RESULTS AND DISCUSSION

Table I shows the distribution of carbon in 4 different samples of molasses and corn steep liquor. All determinations were made on the wet material without any previous treatment. Total carbon in molasses varied from 24 to 30%, of which 17–20% was sugar carbon while 6–11% was non-sugar carbon. Roughly speaking, $\frac{2}{3}$ of the total carbon was sugar carbon and $\frac{1}{4}$ – $\frac{1}{3}$ was non-sugar carbon. Hawaiian cane and Ovid beet molasses contained about twice as much non-sugar carbon as Mason City and Lansing beet molasses. Sperber (7) found that raw sugar molasses contained 31% total carbon, of which 61% was present as sucrose and the remainder (39%) was made up of unknown organic compounds. He reported that the refinery molasses contained some-

TABLE I
Distribution of Carbon in Molasses and Corn Steep Liquor

Materials	Carbon, g./100 g. molasses		
	Total	Sugar	Non-sugar
Hawaiian cane molasses	29.9	19.3	10.6
Mason City beet molasses	26.7	20.0	6.7
Ovid beet molasses	28.9	17.4	11.5
Lansing beet molasses	24.2	18.6	5.6
Corn steep liquor	12.9	2.2	10.7

what less non-sugar compounds. According to Payne (13), Hawaiian cane molasses contained 0.7% organic nitrogen. Assuming the conventional ratio of carbon to nitrogen in protein, 53:16, 2.3% of the carbon could be present in nitrogen compounds which would leave 8.3% of carbon unassigned to any source.

Table II gives typical results from many combinations tried and shows the effects of nutrients on the yields of yeasts. Previously it was noted (8) that best yields of these yeasts were obtained with molasses solution containing phosphate, urea and corn steep liquor. Since corn steep liquor contained 12.9% carbon (Table I), an effort was made to eliminate this extra unknown carbon from the medium by developing a medium that would give a high yield of yeast without corn steep liquor. In evaluating the data, differences of less than 15% are not regarded as being significant (see Table IV for reproducibility of runs). The addition of $(\text{NH}_4)_2\text{HPO}_4$ and KH_2PO_4 to the molasses solution markedly improved the yield of *C. arborea*, while the other two yeasts were only slightly benefited. The addition of corn steep liquor together with $(\text{NH}_4)_2\text{HPO}_4$ and KH_2PO_4 gave the highest yields of *S. cerevisiae* and *C. arborea*. However for *T. utilis* a number of media gave yields as high as the one containing corn steep liquor. For *S. cerevisiae*, ash of corn steep liquor and MgSO_4 were beneficial but MgSO_4 was somewhat superior. For *T. utilis* the ash was only a partial substitute but MgSO_4 completely replaced corn steep liquor. *C. arborea* remained unaffected by the ash or MgSO_4 . Because of the failure to get maximum yields of *S. cerevisiae* and *C. arborea* without corn steep liquor, a number of growth factors were tried in various combinations. Asparagine or

TABLE II
Effect of Nutrients on Yield of Yeasts
 (Mason City beet molasses, 15 hr. fermentation^a)

No.	Additions to molasses medium	Per cent dry yeast, on S. F.		
		<i>S. cerevisiae</i>	<i>T. utilis</i>	<i>C. arborea</i>
1	None	15.4	34.6	30.0
2	0.22% (NH ₄) ₂ HPO ₄ + 0.1% KH ₂ PO ₄	19.2	39.2	47.6
3	0.22% (NH ₄) ₂ HPO ₄ + 0.1% KH ₂ PO ₄ + 0.33% CSS	50.7	64.2	63.2
4	0.22% (NH ₄) ₂ HPO ₄ + 0.1% KH ₂ PO ₄ + 0.33% CSS ash	28.7	50.6	46.9
5	0.22% (NH ₄) ₂ HPO ₄ + 0.1% KH ₂ PO ₄ + 0.25% MgSO ₄	32.5	62.1	44.9
6	No. 5 + 0.1% asparagine	33.2	63.8	55.4
7	No. 5 + 40 γ/l. biotin	34.2	56.7	54.6
8	No. 5 + 10 mg./l. inositol	34.6	59.1	45.5
9	No. 5 + 4 mg./l. pantothenate	28.9	58.1	45.0
10	No. 5 + 10 mg./l. inositol + 4 mg./l. pantothenate	30.0	59.7	51.4
11	No. 10 + 40 γ/l. biotin	36.0	61.3	56.6
12	No. 10 + 40 γ/l. biotin + 0.1% asparagine	34.4	59.3	55.9

^a S. F. denotes sugar fermented and CSS refers to corn steep solids.

biotin gave a marked improvement in the yield of *C. arborea*, but had no effect on the yields of the other two yeasts. No appreciable effect was produced with any yeast by adding inositol and pantothenate individually or combined.

Utilization of Non-Sugar Carbon of Different Molasses

In Table III the utilization of non-sugar carbon by yeasts grown for 14-16 hr. on four kinds of molasses is shown. The data indicate that the kind of molasses had no marked influence on the utilization of non-sugar carbon, except in one case, where *S. cerevisiae* utilized only 7.4% non-sugar carbon of Mason City molasses. The low utilization may be explained as due to a low yield of this yeast on this molasses. For *S. cerevisiae*, of the 4 molasses samples tested, Mason City beet molasses proved poorest as regards yield and utilization of non-sugar carbon. Excluding this exception, utilization of non-sugar carbon ranged from

TABLE III
Utilization of Non-Sugar Carbon by Yeasts Grown on Four Kinds of Molasses
(14-16 hr. fermentation^a)

Yeast fermentation	Yield on S. F.	Carbon, mg./100 ml.			Non-sugar carbon used
		Total	Sugar	Non-sugar	
	<i>per cent</i>				<i>per cent</i>
Lansing beet molasses					
Unfermented	—	475.5	330.0	145.0	—
<i>S. cerevisiae</i>	45.6	130.7	7.3	123.4	15.0
<i>T. utilis</i>	62.5	106.9	10.0	96.9	33.0
<i>C. arborea</i>	56.4	118.8	9.0	109.8	24.2
Mason City beet molasses					
Unfermented	—	507.0	14.0	135.0	—
<i>S. cerevisiae</i>	36.5	139.0	14.8	125.0	7.4
<i>T. utilis</i>	64.9	106.4	24.5	91.6	32.1
<i>C. arborea</i>	57.5	126.7	24.5	102.2	24.3
Ovid beet molasses					
Unfermented	—	510.9	382.0	128.9	—
<i>S. cerevisiae</i>	41.9	122.7	14.4	108.3	15.8
<i>T. utilis</i>	59.5	104.9	12.4	92.5	28.2
<i>C. arborea</i>	57.0	117.1	19.2	97.9	24.1
Hawaiian cane molasses					
Unfermented	—	570.0	372.0	198.0	—
<i>S. cerevisiae</i>	45.4	202.0	32.8	169.2	14.5
<i>T. utilis</i>	63.2	168.0	32.0	136.0	31.3
<i>C. arborea</i>	60.2	178.2	32.0	146.2	26.1

^a The medium was combination No. 10, Table II.

14.5 to 15.8% by *S. cerevisiae*, 28.2-33.0% by *T. utilis* and 24.1-26.1% by *C. arborea*.

Interrelations of Sugar, Alcohol and Non-Sugar Carbon to Yeast Production

In an experiment set up to determine the rate of utilization of non-sugar carbon, it was discovered that early in the fermentation, *e.g.*, 4-8 hr., not only was there no decrease in non-sugar carbon, but there was an actual increase over that contained in the medium at the start. Analysis of the culture for alcohol showed that this accounted for the increase in non-sugar carbon. This finding was unexpected, as it was assumed that, at the low concentration of sugar used, and with the

vigorous aeration given the medium, there would be no alcohol accumulation. The problem was then investigated systematically. Table IV and Figs. 1-6 show the interrelations of yeast growth, sugar consumption, production and utilization of alcohol, and utilization of carbon compounds other than sugar and alcohol. In Figs. 3-6 the alcohol carbon is multiplied by 2 to make the curve more readable. The data deal with the carbon metabolism of 3 different yeasts on 2 kinds of media.

TABLE IV
Reproducibility of Results at Different Times
(Hawaiian cane molasses)

Yeast	Time	CSS medium ^a						G. F. medium ^b		
		Run 1	Run 2		Run 3					
		Yield on S. F.	Yield on S. F.	NSC ^c used	Yield on S. F.	C ₂ H ₅ OH	NSC ^d used	Yield on S. F.	C ₂ H ₅ OH	NSC ^d used
<i>S. cerevisiae</i>	hr.	per cent	per cent	per cent	per cent	mg/100 ml	per cent	per cent	mg/100 ml	per cent
	4	29.9	24.8	0	25.2	215.6	1.6	20.9	186.7	0
	8	32.9	31.9	0	32.7	231.3	15.8	21.1	229.6	1.9
	16	50.4	48.8	19.4	46.2	12.4	16.4	42.5	38.9	17.9
	24	54.9	51.6	21.0	48.4	10.3	19.9	46.2	4.8	19.2
	40	51.1	50.1	20.3	45.3	7.6	13.4	42.0	3.9	8.9
<i>T. utilis</i>	4	43.8	48.0	10.7	49.5	57.1	19.0	44.1	66.8	13.5
	8	57.9	60.2	33.7	54.6	6.4	20.2	58.3	14.9	30.3
	16	61.4	53.3	30.0	59.2	4.7	37.4	58.3	4.4	30.6
	24	59.9	49.5	25.8	55.1	4.3	33.6	57.1	3.9	27.1
	40	60.1	49.5	25.8	53.2	4.0	34.5	52.4	2.6	23.8
<i>C. arborea</i>	4	32.5	36.7	0	32.9	15.6	0.5	43.3	11.7	0
	8	47.7	44.8	15.5	45.9	72.4	22.9	45.9	50.0	27.4
	16	61.0	60.1	31.3	65.4	4.1	35.4	57.1	4.8	27.4
	24	59.7	56.1	25.8	55.4	2.9	32.1	56.6	2.0	23.3
	40	56.2	55.0	18.2	55.0	1.9	30.6	55.9	1.6	25.9

^a Medium consisted of molasses equivalent to 0.92-1.06% glucose + 0.22% (NH₄)₂HPO₄ + 0.1% KH₂PO₄ + 0.33% CSS.

^b Growth factor medium consisted of molasses equivalent to 0.95% glucose + 0.22% (NH₄)₂HPO₄ + 0.1% KH₂PO₄ + 0.025% MgSO₄ + 40 γ/l. biotin, + 10 mg./l. inositol, + 4 mg./l. pantothenate.

^c NSC denotes non-sugar carbon and it includes alcohol carbon.

^d NSC denotes non-sugar non-alcohol carbon, i.e., it excludes alcohol carbon.

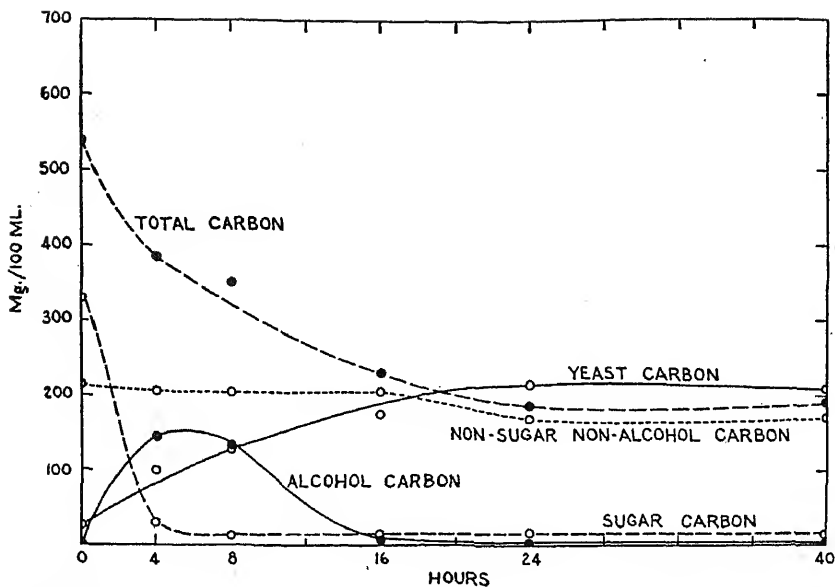


FIG. 1. Utilization of various carbon compounds by *S. cerevisiae*.
(Lansing molasses—urea—CSS medium.)

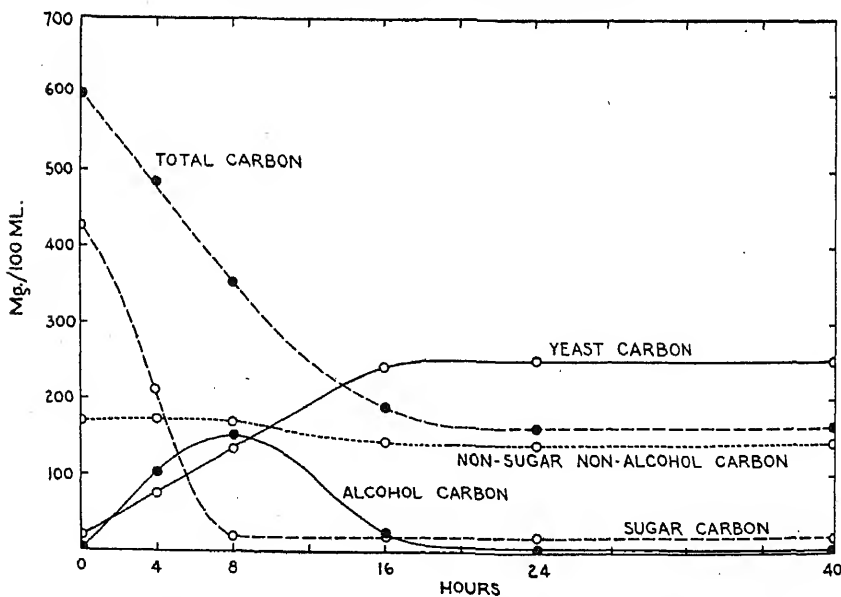


FIG. 2. Utilization of various carbon compounds by *S. cerevisiae*.
(Lansing molasses—(NH₄)₂HPO₄—growth factor medium.)

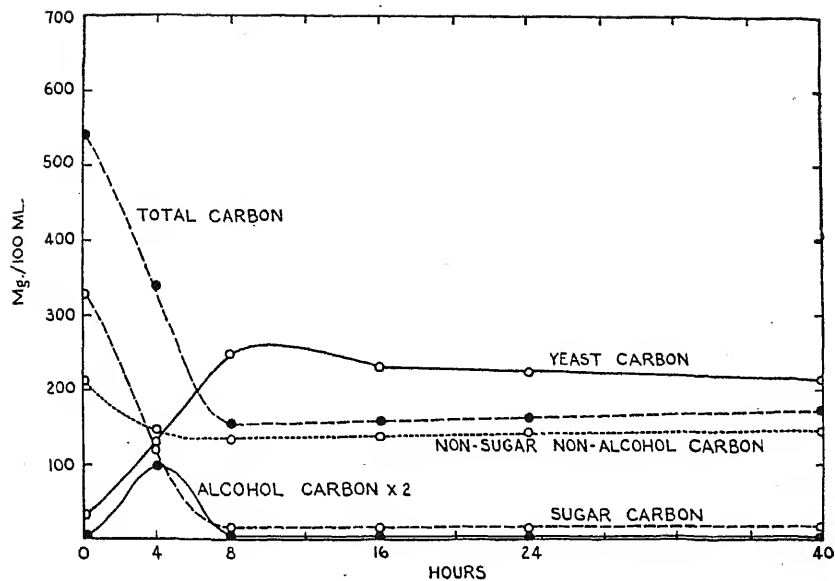


FIG. 3. Utilization of various carbon compounds by *T. utilis*.
(Lansing molasses—urea—CSS medium.)

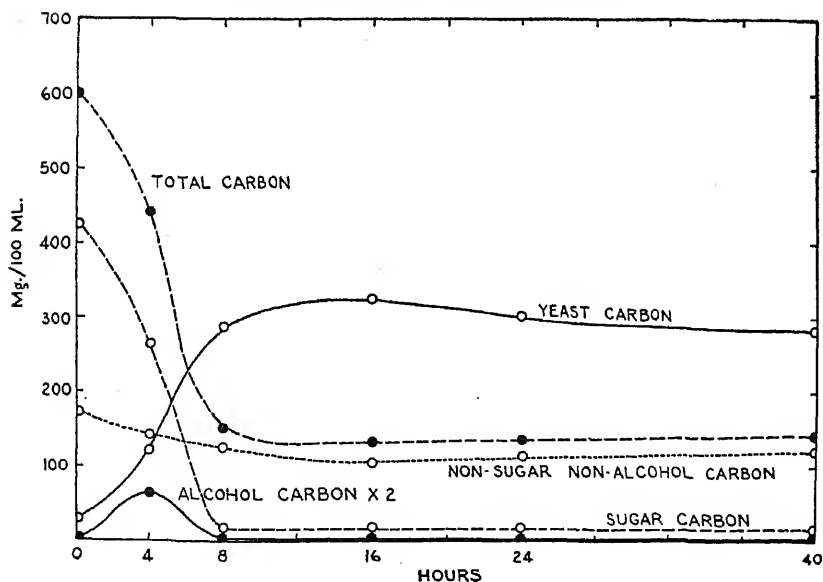


FIG. 4. Utilization of various carbon compounds by *T. utilis*.
(Lansing molasses—(NH₄)₂HPO₄—growth factor medium.)

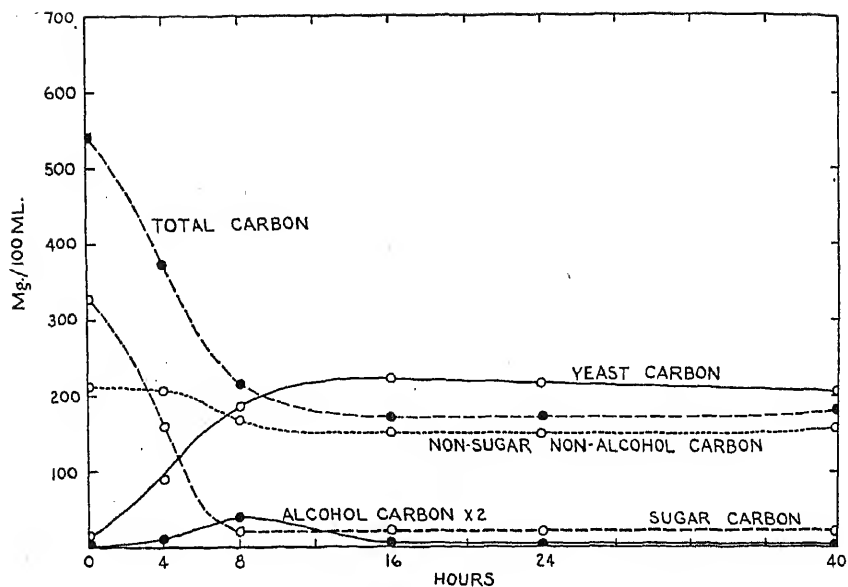


FIG. 5. Utilization of various carbon compounds by *C. arborea*.
(Lansing molasses—urea—CSS medium.)

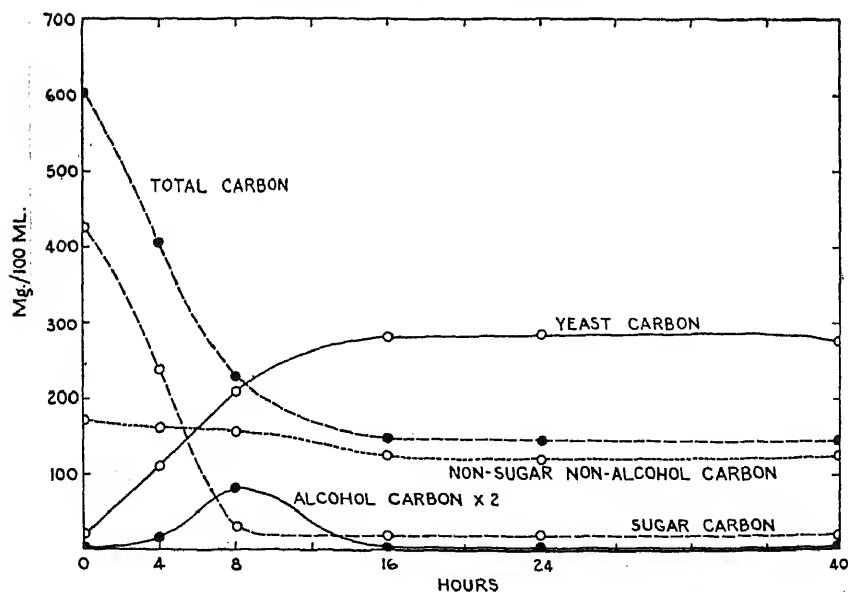


FIG. 6. Utilization of various carbon compounds by *C. arborea*.
(Lansing molasses— $(\text{NH}_4)_2\text{HPO}_4$ —growth factor medium.)

Attention is first called to the sugar utilization and yield of cells. In this discussion, weight of yeast cells (Table IV) and yeast carbon (Figs. 1-6) will be used interchangeably. The actual weight of yeast cells can be readily calculated from the weight of yeast carbon and *vice versa*. The dried yeast was assumed to contain 45.9% carbon (7). From 90 to 96% of the sugar present was fermented in 8 hr. The residual sugar was probably due to the presence of unfermentable reducing substances that are well known to occur in molasses (14,15,16). The maximum yields of cells were obtained in 8 hr. with *T. utilis*, in 16 hr. with *C. arborea*, and in 24 hr. with *S. cerevisiae*. Maximum yields were not reached, however, by the time the sugar had been fermented. Weight of cells continued to increase steadily long after the fermentable sugar had disappeared. For example, Fig. 1 shows that the sugar was gone at 5 hr., but weight of yeast was less than half of that found at 24 hr. Fermentable sugar lasted for 8 hr. in the second *S. cerevisiae* fermentation (Fig. 2), but again only about 50% of the final yield had been reached at the end of 8 hr. The differences are less marked for *T. utilis* and *C. arborea*. Nearly maximum yields were reached by the time the fermentable sugar was consumed.

The curves for alcohol show an interesting relationship to sugar consumption and cell yields, particularly in the case of *S. cerevisiae*. Alcohol increased as long as there was any fermentable sugar left but then fell off rapidly. Meanwhile, the weight of yeast continued to rise until the alcohol disappeared and the non-sugar, non-alcohol carbon reached a constant level. The obvious conclusion from these data is that yeast cells are in part produced from alcohol and other non-sugar compounds.

The quantity of alcohol produced ranged from 230-312 mg./100 ml. with *S. cerevisiae*, from 64-99 mg. with *T. utilis*, and from 38-80 mg. with *C. arborea*. The peak concentration of alcohol was reached in 8 hr. in most cases with *S. cerevisiae* and *C. arborea*, and in 4 hr. with *T. utilis*. The quantity of alcohol produced by *S. cerevisiae* under these markedly aerobic conditions was about $\frac{1}{2}$ of that formed under anaerobic conditions; 460 mg. of ethyl alcohol were obtained/g. of glucose in an anaerobic fermentation of the same medium (G. F. Table IV) as was used for the aerobic production of yeast. In Table V are given yields of ethyl alcohol that have been reported by other investigators as being formed in aerobic production of yeast. Some of the figures reported are even higher than those found by the authors. These figures

TABLE V
Reports on Aerobic Production of Ethyl Alcohol by Yeasts

Yeast	Substrate	Sugar conc. (glucose)	Aeration ^a	Time	Yield on S.F.	C ₂ H ₅ OH	Per cent of anaerobic yield ^b	Reference
		per cent	1/1/m	hr.	per cent	mg./100 ml.		
<i>S. cerevisiae</i>	Grain-wort	2.76	High aeration	22	18.1 ^c	948.0	74.6	(17)
<i>T. utilis</i>	Beet molasses	1.21	6.6	11	41.3 ^c	13.3	2.3	(7)
<i>S. cerevisiae</i>	Beet molasses	2.33	6-8	8	23.5 ^c	800.0	74.6	(18)
<i>S. cerevisiae</i>	Beet molasses	1.07	high aeration	8	24.2	312.5	62.2	Authors
<i>T. utilis</i>	Beet molasses	1.07	High aeration	4	49.0	63.9	12.7	Authors
<i>C. arborea</i>	Beet molasses	1.07	High aeration	4	42.0	79.7	15.8	Authors

^a 1/1/m denotes l. of air/l. of medium/min.

^b Calculated on the assumption that 90% of the theoretical yield is obtained under anaerobic conditions.

^c The figures denote per cent dry yeast on sugar supplied.

are probably due to higher concentration of sugar and lower aeration than were used in our experiments.

That the alcohol was utilized by the yeast and not lost from the medium is shown by the following data: At the peak production of alcohol, if yeast growth was stopped in some of the flasks by adding 25 ml. *N*/1 acid, and the flasks were further shaken for 32-36 hr., 76-84% of the alcohol present at the time of adding the acid was recovered (Table VI), and no increase in the weight of yeast cells was observed. In other words, loss of alcohol from the fermentation medium

TABLE VI
Loss of Ethyl Alcohol by Aeration as Contrasted to Its Utilization
During Fermentation
(Hawaiian cane molasses, urea + CSS medium)

Yeast	Alcohol, mg./100 ml.					Per cent alcohol lost	
	Samples not acidified			Samples acidified			
	4 hr.	8 hr.	40 hr.	40 hr. ^a	40 hr. ^b	4-40 hr.	8-40 hr.
<i>S. cerevisiae</i>	215.5	231.3	7.6	169.0	194.5	21	16
<i>T. utilis</i>	55.1	6.3	4.3	41.5	—	24	—
<i>C. arborea</i>	15.6	72.4	1.9	—	57.1	—	21

^a Sample was acidified with 25 ml. 1 *N* H₂SO₄ after 4 hr.

^b Sample was acidified with 25 ml. 1*N* H₂SO₄ after 8 hr.

due to aeration ranged from 16–24%. Loss of ethyl alcohol in equal concentration in water solution during the same period of aeration was also determined and was about the same order of magnitude.

That yeast can assimilate ethyl alcohol has been reported by a number of investigators (7,19,20). Balls and Brown (21) and Brown and Balls (18), studying the growth of *S. cerevisiae* in aerated beet molasses-mineral salts medium, found that, in about 8 hr. a considerable amount of ethyl alcohol was produced (Table V) which was maximum at the time of sugar disappearance. Later alcohol gradually decreased and CO₂ evolution increased.

TABLE VII
Effect of Size of Inoculum on Yield, Alcohol Production and
Utilization of Non-sugar Carbon
(Hawaiian cane molasses^a)

Inoculum by volume	Time	<i>S. cerevisiae</i>			<i>T. utilis</i>		
		Yield on S. F.	C ₂ H ₅ OH	NSC used	Yield on S. F.	C ₂ H ₅ OH	NSC used
<i>per cent</i>	<i>hr.</i>	<i>per cent</i>	<i>mg./100 ml.</i>	<i>per cent</i>	<i>per cent</i>	<i>mg./100 ml.</i>	<i>per cent</i>
5	2	35.1	68.1	9.4	52.7	10.1	6.9
	4	29.5	142.5	10.9	54.1	28.1	29.0
	8	48.1	75.4	29.7	81.6	2.9	45.7
	16	57.3	2.5	32.9	80.6	2.6	44.9
	24	57.3	2.0	32.8	74.1	2.0	39.7
2.5	2	25.9	48.7	10.3	37.8	10.1	7.7
	4	35.1	138.5	15.7	72.5	14.8	8.0
	8	41.9	89.6	35.6	79.0	3.7	41.2
	16	60.0	2.5	32.5	79.0	3.4	41.1
	24	57.5	1.5	29.5	76.3	2.6	41.0

^a Medium consisted of molasses equivalent to 0.45% glucose + 0.1% urea + 0.1% KH₂PO₄ + 0.33% CSS; 25 ml. medium was taken in 500 ml. flasks; NSC denotes non-sugar, non-alcohol carbon.

Attempts were made to stop alcohol production by decreasing the volume of medium from 50–25 ml. and thus increasing aeration, and by reducing the sugar concentration to 0.5%. In spite of these changes, the percentage of alcohol based on sugar fermented remained about the same (compare Table VII with Table IV). Size of inoculum also had no effect on the amount of alcohol produced by *S. cerevisiae* but,

in case of *T. utilis*, decrease of inoculum by one-half, reduced alcohol production about one-half.

Factors Affecting the Utilization of Non-Sugar, Non-Alcohol Carbon

Figs. 1-6 and Table IV indicate that the kinds of molasses seemed to have no appreciable effect on the overall utilization of non-sugar, non-alcohol carbon. With two kinds of molasses it reached 19-21% with *S. cerevisiae*, 31-37% with *T. utilis*, and 27-35% with *C. arborea*. Although Hawaiian cane molasses contained about twice as much non-sugar carbon as Lansing molasses (Table I), the maximum amount of non-sugar, non-alcohol carbon utilized by yeasts was almost the same in both kinds of molasses. In most cases, as long as sugar carbon was present, *S. cerevisiae* and *C. arborea* did not utilize non-sugar, non-alcohol carbon to an appreciable extent, but utilized it soon after the disappearance of sugar. *T. utilis* behaved somewhat differently. It utilized a considerable amount of non-sugar, non-alcohol carbon even in the presence of sugar. The utilization of this unidentified carbon increased with increase in yields. During later hours of fermentation, the non-sugar, non-alcohol carbon slightly increased, which was probably due to the autolysis of yeast cells. In general, the utilization of the unidentified carbon was higher with urea and corn steep medium than with $(\text{NH}_4)_2\text{HPO}_4$ and growth factor medium. When the ratio of non-sugar to sugar carbon was changed by decreasing the concentration of sugar to about 0.5% instead of 1%, and keeping the concentrations of urea and corn steep solids the same (*i.e.*, 0.1% urea, 0.33% CSS), the yields of yeasts based on sugar fermented were correspondingly high, and the utilization of unidentified carbon was still higher (Table VII). These figures show how misleading yields of yeast may be when based on sugar fermented.

Size of inoculum had no appreciable effect upon the completeness of sugar utilization, on the maximum growth of yeasts, and on the overall utilization of carbon, although, with large inoculum, yield of cells and carbon utilization was somewhat more rapid during the early hours.

SUMMARY

Three yeasts, *Saccharomyces cerevisiae*, *Torulopsis utilis*, and *Candida arborea*, were grown with aeration on 4 lots of cane and beet molasses

in shaken flasks. Molasses was found to contain 17–20% sugar carbon and 6–12% non-sugar carbon. Suitable media were developed to obtain maximum yields (46–66% of dry yeast based on sugar fermented). Yeast growth and utilization of sugar and non-sugar carbon were determined at different intervals from 4 to 40 hr. Nearly 90–96% of the sugar present was fermented in 8 hr. Yeast growth continued even after the disappearance of sugar. Maximum yields of *T. utilis* (58–66%) were obtained in 8 hr., of *C. arborea* (51–65%) in 16 hr., and of *S. cerevisiae* (46–55%) in 24 hr.

During early hours of fermentation, even under very aerobic conditions, a considerable amount of ethyl alcohol was produced. In 100 ml. of medium containing approximately 1% sugar, it reached 230–312 mg. with *S. cerevisiae*, 64–99 mg. with *T. utilis*, and 38–80 mg. with *C. arborea*. Under anaerobic conditions about 460 mg. of ethyl alcohol were obtained from 1 g. glucose with *S. cerevisiae*. Hence, even under the highly aerobic conditions of these experiments, 50% or more of the anaerobic yield was obtained with this yeast. The alcohol was later utilized by the yeast cells.

Utilization of non-sugar carbon increased with increase in yields and reached 21% with *S. cerevisiae*, 37% with *T. utilis*, and 35% with *C. arborea*.

It is evident that non-sugar compounds of molasses contribute materially to the yield of yeasts. To calculate the yield on the basis of sugar, therefore, cannot give a correct understanding of the situation.

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Observations on an Unknown Growth Factor Essential for *Tetrahymena geleii*

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INTRODUCTION

The nutrition of *Tetrahymena geleii* has been extensively investigated by Kidder and his associates. Recent papers by Kidder and Dewey (1,2) showed that *T. geleii* could be grown on a medium containing casein hydrolyzate, the known vitamins, purines, pyrimidines, plus a Norit filtrate from liver extract. This liver fraction furnished an unknown essential factor for *T. geleii* which Dewey (3) designated as Factor II.

We have investigated the properties of this unknown growth factor and have shown that it is made up of 2 or more components. This communication deals with the separation of these two factors and with methods obtained for the concentration of them.

EXPERIMENTAL

Methods

The basal medium employed had the following composition.

Substance	Amount/500 ml. of double strength medium
Dextrose	2.0 g.
Hydrolyzed casein	5.0 g.
DL-Tryptophan	0.2 g.
Hydrolyzed yeast nucleic acid	.25 g.
K ₂ HPO ₄	.1 g.
MgSO ₄ ·7H ₂ O	.1 g.
CaCl ₂ ·2H ₂ O	.05 g.
FeCl ₃ ·6H ₂ O	1.25 mg.
MnCl ₂ ·6H ₂ O	.05 mg.
ZnCl ₂	.05 mg.

Substance	Amount/500 ml. of double strength medium
Inositol	1.0 mg.
Choline chloride	1.0 mg.
Calcium pantothenate	.1 mg.
Thiamine HCl	.1 mg.
Nicotinamide	.1 mg.
Riboflavin	.1 mg.
Pyridoxine HCl	.1 mg.
Pteroylglutamic acid	.1 mg.
Biotin	.05 γ
Final pH of medium	6.8 to 7.0

The casein hydrolyzate was prepared by refluxing 100 g. of Labco casein for 12 hr. with 1000 ml. 6.0 *N* HCl and evaporating the acid *in vacuo*. The solution was neutralized to pH 3.0, diluted to 100 mg./ml., filtered and treated twice with two 10 g. portions of Darco G-60 activated charcoal/l. The pyrimidine and purine supplement was prepared by hydrolyzing 10 g. of yeast nucleic acid for 1 hr. with 50 ml. water + 5 ml. concentrated NH_4OH according to the method of Levene and Bass (4).

The silver precipitate fractions were prepared from a papain digest of the coagulum obtained by cooking ground liver and removing the liquid in a press. This preparation is termed liver-press-cake digest. Ten g. of this was dissolved in 1000 ml. of water, adjusted to pH 3.0, and the proteinaceous precipitate removed. The filtrate was adjusted to pH 2.0 and excess AgNO_3 added. The precipitate was removed and freed of silver with HCl. This fraction was designated the "acid silver precipitate." The filtrate from the pH 2.0 silver precipitation was adjusted to pH 4.0, and a second precipitate collected and freed of silver. The pH 4 filtrate was freed of silver with chloride ion and this fraction was designated the "silver filtrate." To prevent formation of colloidal silver chloride it was sometimes necessary to add HCl, which flocculated the silver chloride precipitate.

The organism used in these studies was *T. gelei* W which has been described by Kidder and Dewey (5). The inoculum was carried in the basal medium containing 1 mg./ml. of liver-press-cake digest. This furnished enough of this fraction for $\frac{2}{3}$ maximum growth. One drop of this inoculum was used directly, without washing, /10 ml. of medium. This heavy inoculum permitted maximum growth in the positive control in 44 hr. The incubation temperature was 25°C. Growth was materially slower at 22° or 30°, and stopped at 37°C.

The assay was carried out as follows: The samples, plus sufficient water to give a total volume of 5 ml., were placed in 50 ml. Erlenmeyer flasks and sterilized by autoclaving 15 min. at 120°C. The medium prepared at double strength was sterilized separately, cooled, and 10 ml. of inoculum added/l. of this medium. This corresponded to 0.05 ml. of inoculum per flask. Five ml. of this inoculum medium were delivered to each flask aseptically with an automatic pipetting machine.

Individual addition of 0.05 ml. of inoculum to each flask has the disadvantage that the free swimming *T. gelei* tend to stratify in the pipette making uniform inoculation difficult. In the present method reproducibility of the assay has been enhanced by addition of the inoculum to the entire batch of medium which may be kept agitated while it is being delivered to the individual flasks.

At the end of the assay period the organisms were killed by the addition of 1 drop of 30% formaldehyde, and the optical density was determined in a photoelectric colorimeter at 600 m μ . If the protozoa were not killed, the optical density fluctuated during the reading, due to the stratification of the living organisms.

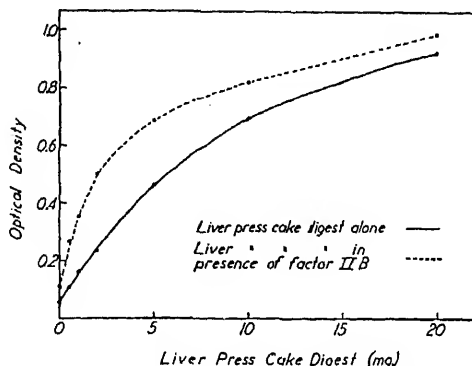


FIG. 1. Growth response produced by liver-press-cake digest in presence and absence of Factor IIB.

Separation of Factor II into Two Fractions, IIA and IIB

During fractionation with heavy metals, loss of activity was observed and recovery of activity was obtained by recombining the fractions. This indicated a resolution into

TABLE I
Supplementary Effect of a Silver Precipitate and a Silver Filtrate

Liver-press-cake digest	pH 2 Silver precipitate	pH 4 Silver filtrate	Growth	Recovery of original activity	Supplementation ratio ^a
mg./10 ml.	mg. \approx /10 ml.	mg. \approx /10 ml.	optical density	per cent	
—	—	—	.02		
2.5			.22		
5.0			.45		
10.0			.71		
20.0			.89		
30.0			1.01		
	10.0		.06	6	
	50.0		.10	2	
		10.0	.22	25	
		50.0	.51	12	
	10.0	10.0	.48	54	1.7
	50.0	50.0	.98	56	4.0

^a Supplementation ratio = $\frac{\text{Observed activity of recombined fractions}}{\text{Sum of activity of component parts}}$

2 separate components. Dried liver-press-cake digest was used as the reference assay standard and as the starting material for the initial fractionation experiments. The response of the organism to this supplement is shown in Fig. 1.

The greatest degree of supplementation was obtained with an acid silver precipitate and a silver filtrate. The silver precipitate alone was approximately 2-6% as active as the starting material, while the filtrate was 12-25% as active. Recombination of these two fractions produced from 2-4 times the effect expected on the basis of the sum of the 2 fractions. The results of this experiment are presented in Table I.

To temporarily designate these two components, that present in the silver precipitate has been termed factor IIA, and that in the silver filtrate factor IIB.

In subsequent assays for factor IIA, the medium was supplemented with a source of IIB, such as the silver filtrate of liver-press-cake digest or a filtrate obtained after precipitation of an alcohol-soluble fraction of liver with saturated ammonium sulfate in aqueous solution. This latter preparation proved to be a more satisfactory source of IIB, and, at a level equivalent to 15 mg. of 80% soluble fraction/10 ml. medium, in the absence of IIA it gave a growth with an optical density of 0.12. This amount contributed sufficient IIB to give a growth of 0.80 with excess of IIA. Since Kidder and

TABLE II
*Separation of Factor II into Two Chemically Distinct Forms by
Chromatographic Adsorption*

Column: 58 g. 300 mesh Florosil,¹ 12 g. Celite filter aid in a column 4.5 cm. diam. X 8 cm. high. Column prewashed with 0.005 *N* acetic acid. 5 g. of liver Norit eluate in 100 ml. water added, followed by eluants as shown in table. The 0.1 *N* NH_4OH was added at beginning of collection of Fraction 8.

Fraction	Solution put through column	Percolate ml.	pH of percolate	Color of percolate	Total units in fraction
1	Sample	70	5.5	—	0
2	0.005 <i>N</i> acetic acid	93	6.0	+++	3600
3	0.005 <i>N</i> acetic acid	112	6.4	++++	5600
4	0.005 <i>N</i> acetic acid	96	6.8	++	1300
5	0.005 <i>N</i> acetic acid	101	6.9	+	300
6	0.005 <i>N</i> acetic acid	99	6.9	±	190
7	0.005 <i>N</i> acetic acid	100	7.1	±	190
8	0.1 <i>N</i> NH_4OH	104	7.0	±	180
9	0.1 <i>N</i> NH_4OH	103	7.4	+	280
10	0.1 <i>N</i> NH_4OH	77	7.4	++	2400
11	0.1 <i>N</i> NH_4OH	59	9.4	++++	3200
12	0.1 <i>N</i> NH_4OH	60	9.8	++++	2000
13	0.1 <i>N</i> NH_4OH	45	10.0	++	300
14	0.1 <i>N</i> NH_4OH	64	10.3	+	71
Total recovery					20,000
Starting material					24,000

Dewey (6) has shown that the IIB supplement can be replaced by copper ion and pyridoxal, it seems unnecessary to describe in detail the preparation of the IIB supplement by ammonium sulfate precipitation. A standard unit of factor IIA for assay purposes was arbitrarily taken as the amount present in one mg. of liver-press-cake digest. The response curve of this standard in the presence of a IIB supplement is shown in Fig. 1.

Demonstration of Two Chemically Different Forms of Factor IIA

During attempts to purify factor IIA by chromatographic adsorption, evidence accumulated that 2 forms of the factor with differing chemical properties were present.

TABLE III
Properties of Factor IIA

Treatment	Recovery of activity per cent
Stability experiments	
Starting material. Liver Norit eluate, 4.2 units/mg.	100
8.0 N sulfuric acid 1 hr. 120°C.	16
1.0 N hydrochloric acid 1 hr. 120°C.	79
0.1 N hydrochloric acid 1 hr. 120°C.	90
pH 6 1 hr. 120°C.	114
0.2 N sodium hydroxide 1 hr. 120°C.	105
2.0 N sodium hydroxide 1 hr. 120°C.	79
Lead precipitation	
Starting material Liver Norit eluate, 4.2 units/mg.	100
Filtrate from first lead precipitation at pH 7	26
Precipitate: Redissolved at pH 3 with nitric acid	
Insoluble lead salts at pH 3	48
Soluble fraction pH 3.0 neutralized to pH 7.0 to reprecipitate lead salts	
Precipitate of lead salts insoluble at pH 7	17
Filtrate of materials soluble with lead at pH 7	5
Saturated ammonium sulfate precipitation	
Starting material. Liver Norit eluate ³ , 4.2 units/mg.	100
Filtrate	1.2
Precipitate	100
Extraction of aqueous solutions with immiscible solvents	
Starting material. pH 3.0 filtrate of papain digest of liver Norit eluate, 1.1 units/mg. of original eluate	100
Butanol extraction pH 3	
Butanol phase	73
Aqueous phase	35
Amyl acetate extraction pH 3	
Amyl acetate phase	8
Aqueous phase	95
Chloroform extraction pH 3	
Chloroform phase	9
Aqueous phase	78

One form was contained in a band which moved rapidly on a Florosil¹ column at pH 6.0-7.0, while the other was adsorbed under these same conditions, and moved rapidly when the column was developed with 0.1 *N* ammonium hydroxide. These two forms are biologically identical for *T. gelei* but have different chemical properties. The starting material used in this experiment was a liver Norit eluate, and the results obtained are summarized in Table II.

Properties of Factor IIA

The stability of factor IIA, and some of its chemical properties are shown in Table III. The results show that this factor is stable to hydrolysis at 120°C. with 2.0 *N* NaOH or 1.0 *N* HCl. The only procedure which destroyed the activity was hydrolysis with 8.0 *N* sulfuric acid at 120°C. Precipitation with lead acetate resulted in only partial precipitation of the activity. Reprecipitation of the pH 7.0 lead precipitate by dissolving at pH 3.0 and reprecipitating at pH 7.0 yielded most of the activity in the lead precipitate. This showed that one form of IIA is readily precipitated by lead while the other is not. This constitutes further evidence for the existence of 2 factors with the same biological activity. The partition of activity with other fractionation procedures was probably the result of this multiplicity of forms.

Extraction with immiscible solvents showed that the activity can be extracted at pH 3.0 by butanol but not by amyl acetate or chloroform.

Distribution of Factor IIA in Natural Products

A variety of natural products were assayed for Factor IIA. The results are presented in Table IV.

TABLE IV
Factor IIA Content of Natural Materials

Material	Unit/g.
Liver-press-cake digest (reference standard)	1000
Alfalfa leaf meal	500
"Cerophyll"	2000
Yellow corn	20
Corn steep liquor (dried)	350
Soybean meal	200
Buttermilk (dried)	20
Molasses	10
Tomato juice (liquid basis)	8
Yeast extract (Difco)	500
Yeast (Anheuser-Busch strain G dried)	160
Autolyzed beef liver (wet basis)	130
Autolyzed pork pancreas (wet basis)	430
Pork muscle	28

¹ Manufactured by the Floridin Co., Inc., Warren, Penna.

DISCUSSION

The available evidence indicates that Factor IIA is not identical with any of the known vitamins. The following compounds also have proven inactive, pimelic acid, 2-methyl-1,4-naphthoquinone, ascorbic acid, thioglycollic acid, vaccenic acid,² oleic acid, Tween 80,³ creatine, creatinine, and indoleacetic acid. A streptogenin concentrate consisting of trypsin-digested casein (7) was inactive. Factor IIA is differentiated from the "animal protein factor" for chicks by the fact that fractions of IIA were inactive when assayed with chicks on a diet consisting primarily of 70% soy bean meal and 23% yellow corn meal and containing all the other known vitamins. Chicks on this diet showed a growth response to concentrates of the "animal protein factor" (8) or the "cow manure factor" (9). Factor IIA concentrates were found to be inactive for *Lactobacillus lactis* Dorner under conditions in which this organism gave a response to concentrated anti-pernicious anemia liver extracts which presumably functioned as a source of "vitamin B₁₂" (10).

In view of the essential nature of IIA for the growth of a protozoon, the name "Protogen" is suggested as a tentative name until a more suitable name, based on chemical structure, can be formulated.

ACKNOWLEDGMENT

The authors are indebted to Dr. George W. Kidder for a culture of this organism, and for valuable assistance received during the course of these experiments.

SUMMARY

(1) An unknown growth factor for *Tetrahymena geleii* W, "Factor II" was separated into two components designated Factor IIA and Factor IIB.

(2) Factor IIA was concentrated from various liver fractions. It was found to exist in two forms with differing chemical properties as shown by adsorption and precipitation procedures.

(3) The distribution of factor IIA in certain natural materials is reported.

² Vaccenic acid samples obtained through the courtesy of Dr. Klaus Hofmann and Dr. E. E. Snell.

³ Tween 80 is a sorbitol ester of oleic acid, and is manufactured by Atlas Powder Co., New York, N. Y.

(4) Factor IIA, for which the name "Protogen" is suggested, was not replaceable by 2-methyl-1,4-naphthoquinone, ascorbic, pimelic, thioglycollic, vaccenic or indoleacetic acids, Tween 80, creatine, creatinine, or a "strepogenin" concentrate. Concentrates of Factor IIA would not replace the "animal protein factor" in the chick assay, and did not stimulate the growth of *L. lactis* Dorner.

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The Effect of Various Compounds on Adaptive Enzyme Formation in Mycobacteria

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INTRODUCTION

Certain mycobacteria rapidly form adaptive enzymes for the oxidation of benzoic acid (1) and this formation, which occurs in non-proliferating cells, is inhibited by streptomycin. Spiegelman (2) and Monod (7) have shown that a number of metabolic inhibitors prevent the formation of adaptive enzymes in yeasts. Apparently, enzyme formation requires energy, and any compound which interferes with energy-yielding reactions may, therefore, inhibit enzyme formation. The effectiveness of any compound will depend in part on the metabolic pattern of the particular cell and the relation of the reaction inhibited to the synthetic processes involved in enzyme formation. It takes, for instance, 20 times more streptomycin to inhibit enzyme formation in one species of mycobacteria than in another, but the relationship is reversed when certain other drugs are used. The following describes the effect of various drugs and conditions on enzyme formation in *Mycobacterium lacticola* and *Mycobacterium BCG* No. 8240.

EXPERIMENTAL

M. lacticola, a soil mycobacterium originally obtained from Dr. Van Niels' collection, was grown 48 hr. at room temperature in a synthetic medium (3) with glucose as sole carbon source. This organism oxidizes benzoic, *p*-hydroxy-, and *m*-hydroxybenzoic acids by specific enzymes (4). Any of these acids can be substituted for the glucose as the sole carbon source for growth, and cells grown in their presence have the specific enzyme already formed. The effect of certain agents on the preformed enzyme was compared with the effects on the formation of the enzyme in normal organisms grown in glucose. In all cases the cells were washed free of the medium by centrifugation and finally suspended in *M*/20 Na-K-phosphate buffer at the desired pH so that there was 0.1 ml. of packed cells/ml. Five-tenths ml. of this suspension was used in each Warburg vessel in a final volume of 2.0 ml.

Table I shows the effect of hydrogen ion concentration on the formation of the enzyme which oxidizes *m*-hydroxybenzoic acid. The enzyme is formed very slowly at pH 6.0 and most rapidly at pH 8.0. The use of a higher pH would entail a different buffer so the maximum rate could not be obtained under comparable conditions. Examination of the values, however, indicates that pH 8.0 is probably near the maximum because the difference in rate of formation between pH 6.7 and 7.8 is small compared with the difference between pH 6.7 and 6.0. The rate of oxidation of *m*-hydroxybenzoic acid by the preformed enzyme varies in the same way with pH but this variation, particularly between pH 6.0 and 6.7, is insignificant compared to the difference in enzyme formation at these two hydrogen ion concentrations. Exactly the same relationship obtains for benzoic and *p*-hydroxybenzoic acid. At all three hydrogen ion

TABLE I

The Effect of Different Hydrogen Ion Concentrations on the Formation of the Adaptive Enzyme for m-Hydroxybenzoic Acid by M. lacticola

For the effect of hydrogen ion concentrations on the activity of the pre-formed enzyme, organisms grown with 100 mg.-% *m*-hydroxybenzoic acid as sole carbon source were used. The control oxygen uptakes (autorespiration) have been subtracted in each case. A minus sign indicates an inhibition of the control uptake. The figures in mm.³ O₂ represent the oxidation of 1.0 mg. (3.6×10^{-3} M) *m*-hydroxybenzoic acid, 37°C.

Time <i>hr.</i>	Normal organisms			Organisms grown in 100 mg.-% <i>m</i> -hydroxybenzoic acid		
	pH 6.0	pH 6.7	pH 8.0	pH 6.0	pH 6.7	pH 8.0
0:30	-2	1	7	112	121	166
1:00	-3	8	17	263	277	315
1:30	0	23	26	389	386	413
2:00	1	38	45	462	436	438
2:30	8	65	72			
3:30	25	128	121			

concentrations, however, the enzyme for *p*-hydroxybenzoic acid is formed most rapidly, that for benzoic acid least. The dissociation constants for benzoic, *m*-hydroxy- and *p*-hydroxybenzoic acids are 6.8, 7.5, and 2.9×10^{-5} , respectively, at 25°C., so there is no correlation between the degree of dissociation and the rate of enzyme formation. Penetration of the molecule into the cell is probably not the limiting factor.

Monod (7) and Spiegelman (2) have shown that 2,4-dinitrophenol, and Reiner (8) that iodoacetate inhibit adaptive enzyme formation in certain yeasts. Table II shows that these 2 compounds are also effective with *M. lacticola*. Fluoroacetate also acts in the same way. Throughout the pH range it is apparently 20 times as effective as iodoacetate. This ratio is difficult to obtain accurately because fluoroacetate inhibits the oxidation of benzoic acid by the preformed enzyme to a greater extent than iodo-

TABLE II

*The Effect of 1.0 γ Sodium Fluoroacetate, (0.5×10^{-6} M) 1.0 γ Dinitrophenol (2.7×10^{-6} M) and 1.0 γ Sodium Iodoacetate (2.4×10^{-6} M) on the Formation of the Adaptive Enzyme for Benzoic Acid by *M. lacticola**

For the effect of these compounds on the preformed enzyme organisms grown with 100 mg.-% benzoic acid as the sole carbon source were used. The control oxygen uptakes (autorespiration) have been subtracted in each case. A minus sign indicates an inhibition of the control uptake. The figures in mm.³ O₂ represent the oxidation of 1.0 mg. sodium benzoate (3.5×10^{-3} M) pH 6.7, 37°C. The drugs were added 60 min. before the benzoate.

Time	Normal organisms				Organisms grown in 100 mg.-% benzoic acid			
	Control	1.0 γ fluoroacetate	1.0 γ dinitrophenol	1.0 γ iodoacetate	Control	1.0 γ fluoroacetate	1.0 γ dinitrophenol	1.0 γ iodoacetate
hr.								
0:30	0	-2	2	0	63	33	61	59
1:00	8	1	6	4	146	65	135	136
1:30	30	7	27	23	224	110	204	209
1:50	57	16	50	46	277	122	258	264
2:35	137	49	128	121	383	192	356	369
3:05	208	83	200	191	454	229	428	433
3:35	275	114	265	255	461	269	459	459
Per cent inhibition		100	—	—		52	3	6
		88	25	50		55	6	7
		77	10	23		51	9	7
		72	12	16		56	7	5
		64	7	12		50	7	4
		60	4	8		50	6	4
		61	3	7		42	0	0

acetate does. It is not possible to determine how much this inhibition contributes to the apparent inhibition of enzyme formation. It should, however, be noted that part of the inhibition in the preadapted cells may also be an inhibition of further enzyme formation. The oxygen uptake in the second half-hour period is always greater than in the first, indicating enzyme formation. In other words, the amount of enzyme in the cells grown in the presence of benzoic acid is not maximal and can be increased when benzoic acid is added to the Warburg vessels. The probable reason for this is that cells grown in benzoic acid first produce enzyme and then lose some as the benzoic acid is used up. That such decay occurs is shown below in experiments with *M. BCG*.

With *M. lacticola*, fluoroacetate inhibits enzyme formation at lower concentrations than 2,4-dinitrophenol at pH 6.7 and 7.8. At pH 6.0 the two drugs are about equally effective. Table III shows the effect of different concentrations of fluoroacetate. As

TABLE III

*The Effect of Different Concentrations of Sodium Fluoroacetate (1×10^{-6} – 1×10^{-5} M) on the Formation of the Adaptive Enzyme for Benzoic Acid by *M. lacticola**

For the effect on the preformed enzyme, organisms grown with 100 mg.-% benzoic acid as the sole carbon source were used. The control oxygen uptakes (autorespiration) have been subtracted in each case. The figures in mm³ O₂ uptake represent the oxidation of 1.0 mg. sodium benzoate (3.5×10^{-3} M) pH 6.7, 37°C. The fluoroacetate was added 60 min. before the benzoate.

Time	Normal organisms					Organisms grown in 100 mg.-% benzoic acids				
	Control	0.1 γ	0.3 γ	0.6 γ	1.0 γ	Control	0.1 γ	0.3 γ	0.6 γ	1.0 γ
hr.										
0:30	2	0	0	0	0	115	90	77	71	66
1:00	3	0	0	0	0	252	202	168	143	127
1:30	11	6	5	0	0	388	323	275	228	191
1:50	17	10	4	0	0	432	385	340	281	233
2:35	55	38	18	7	4	457	424	384	395	360
3:05	93	69	33	16	11					
3:35	139	112	56	30	24					
4:15	219	185	105	56	44					
Per cent inhibition		100	100	100	100		22	33	38	43
		100	100	100	100		20	33	43	50
		45	55	100	100		14	29	41	50
		41	77	100	100		11	22	35	46
		31	67	87	93		17	16	13	43
		26	64	83	88					
		19	60	78	83					
		16	52	74	80					

little as 0.1 γ in 2.0 ml. (0.5×10^{-6} M) still inhibits enzyme formation to an appreciable extent. This sensitivity might indicate that fluoroacetate was interfering directly with the transfer of energy to the enzyme forming system. Such a conclusion does not necessarily follow, because the oxidation of various substrates by this organism is very sensitive to fluoroacetate. Thus 1.0 γ /ml. (1×10^{-6} M) inhibits the oxidation of acetic acid 90% and that of glucose and pyruvate 50% and 30%, respectively. The autorespiration of these washed bacteria is very small, averaging 8–10 mm.³/hr. This is not inhibited by fluoroacetate or iodoacetate, or increased by 2,4-dinitrophenol in the concentrations in which these drugs were used. The latter 2 drugs in low concentrations cause less inhibition of the oxidation of added substrates than fluoroacetate. The possibility still remains that these drugs are interfering with energy transfer rather than energy production. That this is so for 2,4-dinitrophenol is indicated by the work of Loomis and Lipmann (5).

The effect of these 3 drugs on *M. BCG* is apparently different. The organisms, grown for 2 days on Long's medium, with or without 100 mg.-% benzoic acid, and then washed and suspended in the same way as *M. lacticola*, have a high autorespiration which averages 100 mm³/hr. Small concentrations of the drugs have little effect on the autorespiration or on enzyme formation. Beginning at about 50 γ /ml. all 3 compounds increase the autorespiration. For a given number of cells, the effect is maximal at pH 6.0 with a 2,4-dinitrophenol concentration of 100 γ /ml. (5.4×10^{-4} M) and for the other two drugs 500 γ /ml. (5.0×10^{-3} M fluoroacetate and 2.4×10^{-3} M iodoacetate). At these high concentrations there is apparent inhibition of enzyme production, but because of the great increase in the autorespiration it is difficult to interpret these results with certainty. Fig. 1 shows the effect of the 3 drugs on the autorespiration.

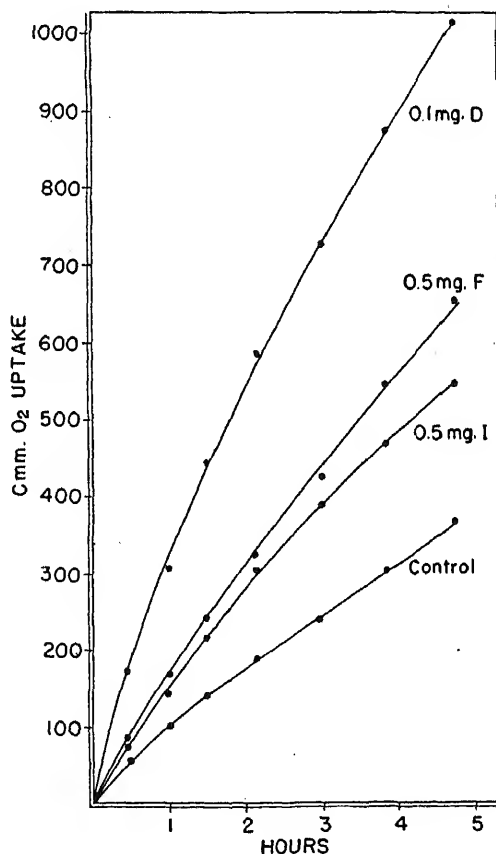


FIG. 1. The effect of 0.5 mg. sodium iodoacetate (1.2×10^{-3} M) (I), 0.5 mg. sodium fluoroacetate (2.5×10^{-3} M) (F), and 0.1 mg. dinitrophenol (2.7×10^{-4} M) (D) on the oxygen uptake of a washed suspension of *M. BCG* at pH 6.0 and 37°C.

Their similar behavior on the autorespiration indicates that one aspect of their mechanism of action may be essentially the same although fluoroacetate and iodoacetate inhibit the oxidation of added substrates, whereas 2,4-dinitrophenol increases the oxidation of the same compounds.

The decay of the adaptive enzyme can be shown quite easily with *M. BCG*. Organisms grown on normal medium were washed and aliquots placed in Warburg vessels. Five γ (1.7×10^{-4} M) sodium benzoate were added to the vessels. This was sufficient, as previously shown (6), to elicit appreciable enzyme formation. At the end of 60 min., 0.5 mg. sodium benzoate was added to one of the vessels and the rate of oxygen uptake measured. At the end of another 60 min., 0.5 mg. benzoate was added to another vessel and the rate measured. This procedure was continued and the results, given in Table IV, show that the amount of enzyme formed as a result of exposure to 5 γ of

TABLE IV

The Decay of the Adaptive Enzyme with Time

A normal strain of *M. BCG* was incubated with 5.0 γ sodium benzoate. At various intervals 0.5 mg. of sodium benzoate was added from the side arm. After 240 min., a definite decrease in the oxidation rate of the benzoate is seen, compared to the rate after 60 min. The control was not incubated with 5.0 γ benzoate. The figures in mm.³ O₂ uptake represent the oxidation of the 0.5 mg. benzoate. (1.7×10^{-3} M) pH 6.7, 37°C.

Time	Control	60' incubation	120' incubation	240' incubation
<i>hr.</i>				
0:15	-4	7	9	-1
0:30	5	29	23	18
1:00	56	119	112	81
2:00	287	296	301	220

benzoate disappears after about 240 min. incubation at 37°C. A similar experiment can be done with cells grown in medium containing benzoate. In this case more enzyme is present and more time is required before the loss becomes measurable. The rate of formation of adaptive enzyme, as well as the rate of oxidation of the benzoic acid by *M. BCG* is independent of the pH in the range 6.0-8.0. Possibly the intracellular pH is not readily altered in this organism.

DISCUSSION

It has previously been shown (1) that streptomycin inhibits the production of adaptive enzymes in the 2 species of mycobacteria discussed above. *M. BCG* was 20 times more sensitive to its effect than *M. lacticola*, and streptomycin was without action on the autorespiration or on the oxidation of added substrates. It seemed possible that strepto-

mycin was combining with some nucleoprotein and thus blocking the actual formation of the enzyme from its precursor. On the other hand *M. lacticola* is much more sensitive to the effect of fluoroacetate, iodoacetate and 2,4-dinitrophenol than is *M. BCG*. In the former species these drugs are without effect on the autorespiration but do inhibit enzyme formation and the oxidation of added substrates. In the latter, autorespiration is greatly increased; fluoro- and iodoacetate inhibit the oxidation of added substrates such as trehalose, glucose, and fatty acids, whereas 2,4-dinitrophenol increases the rate and extent of oxidation of these added compounds. Despite this last difference, it is possible that the 3 drugs may be acting by similar mechanisms.

SUMMARY

1. Fluoroacetate, iodoacetate, and 2,4-dinitrophenol inhibit adaptive enzyme production in *M. lacticola* and probably also in *M. BCG*. Fluoroacetate is the most effective in this respect.

2. These drugs do not affect the autorespiration, which is very small, of *M. lacticola* but inhibit the oxidation of added sugars and fatty acids.

3. Adaptive enzyme production in *M. lacticola* occurs very slowly at pH 6.0, much more rapidly at 8.0. Fluoroacetate and iodoacetate inhibit the production at both pHs equally. 2,4-Dinitrophenol is more active in the acid range.

4. The 3 drugs greatly stimulate the autorespiration of *M. BCG*. The oxidation of added sugars and fatty acids is inhibited by fluoroacetate and iodoacetate but increased by 2,4-dinitrophenol.

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Yeast Nucleic Acid. II. Cultural Characteristics of Yeasts in Nucleic Acid Biosynthesis

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INTRODUCTION

The characteristically high nucleic acid concentration in the cytoplasm of cells undergoing rapid protein synthesis was demonstrated by Caspersson *et al.* (1,2,3,4,5). Caspersson and Brandt (6) and Brandt (7) extended this generalization to yeast. It was shown recently that the nucleic acid content of yeast cells can be regulated (within certain limits) by cultivation under experimental conditions conducive to either rapid or slow biosynthesis of proteins (8).

Since different yeast cultures were observed to vary in nucleic acid content (8), it was considered of interest to extend the work and to seek correlation with cultural characteristics such as growth factors (9). It was previously demonstrated that the percentage of nucleic acid in yeast cells is dependent upon the ammonium and phosphate ion concentrations in the medium (8). Further investigation was required in order to ascertain whether this observation constitutes a general principle for yeasts or depends upon the type of yeast. It was also of interest to determine the nucleic acid contents of different yeasts at successive growth stages.

To facilitate this work the macro method for the determination of nucleic acid in yeast by spectrophotometry (8) was modified for use on semimicro and micro scales.

EXPERIMENTAL

METHOD FOR DETERMINATION OF NUCLEIC ACID IN YEAST

To a weighed sample of yeast¹ (dry weight 10–150 mg.) in a small test tube (13 × 100 mm.) is added 1 ml. of 10% trichloroacetic acid. The mixture is mechanically

¹ Weighed samples of yeast may also be suspended in water with a Waring Blender. Aliquots are centrifuged to remove water and the residues analyzed in the same manner as samples individually weighed.

shaken for 15 min. at room temperature, and centrifuged at 4000–5000 r.p.m. in a Servall Angle Centrifuge. After discarding the supernatant liquid, the residue is twice extracted for 5 min. by shaking with 1 ml. volumes of 10% trichloroacetic acid. The residue is then washed by mixing and centrifuging with 3 ml. of water.

The washed residue is hydrolyzed for 15 min. at 90°C. with 1 ml. of 5% trichloroacetic acid. The mixture is centrifuged after cooling and the solution is decanted into a 200 ml. volumetric flask. The residue is washed at room temperature by shaking for 5 min., first with 2 ml. of 5% trichloroacetic acid, and then with 3 ml. of water. The washings are collected in the 200 ml. flask. One ml. of 40% NaOH solution is added and the contents are diluted to volume with water. The final pH is approximately 11.7.

The optical density is read at 2600Å. against a blank solution consisting of 3 ml. of 5% trichloroacetic acid and 1 ml. of 40% sodium hydroxide diluted with water to 200 ml. Calculations are made from the standard curve (8).

Twelve tubes are run simultaneously for triplicate analyses of 4 yeasts.

In Table I are listed the results obtained on 3 yeasts by macro and micro analyses for nucleic acid.

TABLE I
Comparison of Results from Macro and Micro Analyses for Nucleic Acid

Yeast	Macro	^a %, N. A.	Micro
1	4.63		4.56
2	7.60		7.56
3	3.44		3.52

^a Each analytical value is the average of 3 duplicate analyses on identical yeast samples and is calculated to a dry basis.

Anaerobic Propagation of Yeasts in Molasses

Thirteen cultures of yeast were propagated in molasses without aeration. Yeast yields were determined as well as the nitrogen and nucleic acid contents. These data are recorded in Table II.

Yeast Propagation on Media of High and Low Nitrogen Levels

Five cultures of yeast were propagated without aeration on 2 media. The first medium (high nitrogen) contained 222 g. of molasses, 0.6 g. of ammonium sulfate, and 0.6 g. of phosphoric acid (tech., 75%). The second medium (low nitrogen) contained 111 g. of molasses, 55.5 g. of dextrose, and 0.6 g. of phosphoric acid (tech., 75%).

Table III shows the effects of the nitrogen content of the medium upon the nitrogen and nucleic acid concentrations in the experimental yeasts.

Nitrogen and Nucleic Acid Concentrations in Yeasts at Different Growth Stages

Four yeasts were propagated through 3 successive growth stages (1, no aeration; 2, low aeration; 3, high aeration). In each case the medium consisted of molasses supplemented with excess ammonium sulfate and phosphoric acid. The nitrogen and nucleic acid values obtained at each stage are recorded in Table IV.

TABLE II
Propagation of Yeasts in Molasses without Aeration

Culture	Bios no.	^a %, N	^a %, N. A.	Yeast yield
				<i>per cent</i>
<i>S. cerevisiae</i> Hansen (Commercial Bakers' Yeast)	236	^b 9.17 9.34	7.73 7.22	11.0 9.0
<i>S. cerevisiae</i> Hansen Bakers' Yeast ATCC 2335	234	8.99	7.17	7.7
<i>Candida guilliermondii</i>	3	7.26	6.22	0.7
<i>S. carlsbergensis</i> var. <i>mandshuricus</i> I	235	^b 7.53 8.22	5.04 5.52	10.6 7.6
<i>Torula cremoris</i>	237	^b 8.51 7.89	5.74 5.14	3.4 4.4
<i>Torula spherica</i>	237	8.37	5.27	2.7
<i>Torula utilis</i>	0	6.29	4.61	2.3
<i>Brettanomyces bruxellensis</i>	5	8.02	5.27	1.8
<i>S. cerevisiae</i> Hansen str. <i>anamensis</i>	23	6.93	5.15	8.3
<i>S. cerevisiae</i> Hansen str. Sake	2	8.33	4.53	10.1
<i>Zygosaccharomyces marxianus</i>	7	^b 7.61 8.57	3.88 4.31	4.6 3.9
<i>S. marxianus</i>	0	6.25	3.84	5.2
<i>Zygosaccharomyces lactis</i>	37	6.43	3.36	2.8

^a Calculated to dry basis.^b Two sets of values are given when the entire experiment was repeated.

TABLE III
Effects of Inorganic and Organic Nitrogen Content of Media on Nitrogen and Nucleic Acid Levels of Yeasts Propagated without Aeration

Yeast culture	High N medium			Low N medium		
	^a N	^a N. A.	Yeast yield	^a N	^a N. A.	Yeast yield
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
<i>S. cerevisiae</i> Hansen (Commercial Bakers' Yeast)	9.17	7.73	11.0	7.55	5.82	6.9
<i>S. cerevisiae</i> Hansen Bakers' Yeast ATCC 2335	8.99	7.17	7.7	8.00	6.36	6.1
<i>S. cerevisiae</i> Hansen str. <i>anamensis</i>	6.93	5.15	8.3	6.94	5.07	7.4
<i>S. cerevisiae</i> Hansen str. Sake	8.33	4.53	10.1	6.27	4.54	6.8
<i>Torula cremoris</i>	8.51	5.74	3.4	8.76	5.95	2.8

^a Calculated to dry basis.

TABLE IV
Differences in Nitrogen and Nucleic Acid Concentrations in Various
Yeast Cultures at Different Growth Stages

Growth stage	<i>S. cerevisiae</i> Hansen Commercial Bakers' Yeast		<i>S. carlsbergensis</i> var. <i>mandshuricus</i>		<i>Torula cremoris</i>		<i>Zygosaccharomyces</i> <i>marxianus</i>	
	^a N	^a N. A.	^a N	^a N. A.	^a N	^a N. A.	^a N	^a N. A.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
No aeration	9.26	7.48	7.88	5.28	8.20	5.44	8.09	4.09
Low aeration	7.75	7.06	7.18	4.78	8.13	5.52	7.82	3.72
High aeration	8.80	6.16	7.38	4.96	7.32	4.26	6.89	3.42

^a Calculated to dry basis.

RESULTS AND DISCUSSION

The nucleic acid data obtained by the semimicro techniques corresponded very closely to the results on large samples. The method described above permits convenient analyses of samples containing as little as 300 γ of nucleic acid.

The nucleic acid content of a yeast is a cultural characteristic. The yeasts cultivated under identical conditions contained from 3.36 to 7.73% nucleic acid. There was also a wide range (6.25 to 9.34%) in the total nitrogen of these yeasts. The yeast with the highest nitrogen also contained the most nucleic acid; but no general correlation was evident. The 2 yeasts with greatest amounts of nucleic acid belong to the genus *Saccharomyces*. However, other species in this genus contain considerably less nucleic acid. The strains of the species *Cerevisiae* having bios numbers 236 and 234 showed particularly high nucleic acid levels.

Several yeasts were propagated anaerobically on high and low nitrogen media in an attempt to realize large differences in the nitrogen content of each yeast culture at the same growth stage. Inspection of the data in Table III showed that the nitrogen contents of 3 of the 5 yeasts fell off sharply in the low nitrogen medium. In one of these instances (*S. cerevisiae* Hansen str. Sake) the nucleic acid concentration remained constant whereas the other 2 yeasts also displayed decreases in nucleic acid concentration. It appears probable that the *S. cerevisiae* Hansen str. Sake cultivated on the high nitrogen medium contained a large quantity of incompletely assimilated nitrogenous compounds. In all cases the yeast yields were lower on the low nitrogen medium.

With regard to the effect of the growth stage upon the nucleic acid content of yeasts, the data presented in Table IV indicate that at the higher growth stages (increased aeration) the nucleic acid values generally decreased. These decreases were sharpest with the *S. cerevisiae* and *Torula cremoris* yeasts. Each yeast displayed a different pattern; *i.e.*, different yeasts containing similar amounts of nitrogen did not necessarily contain similar amounts of nucleic acid.

SUMMARY

1. The spectrophotometric method for the quantitative determination of nucleic acid in yeast was adapted to usage on a semimicro or micro scale.

2. The nucleic acid content of yeasts was shown to be a cultural characteristic.

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The Effect of Amino Acid Antagonists on Respiration of *Escherichia coli*¹

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INTRODUCTION

Growth-inhibitory effects of specific metabolite antagonists of the vitamin and amino acid types such as pyridine-3-sulfonic acid (1), thienylalanine (2), and allylglycine (3), have been repeatedly demonstrated. However, detailed knowledge of the mechanism of these inhibitions is only fragmentary at the present time. In an attempt to gain further insight regarding such action, a study of the respiration of *Escherichia coli* under the influence of various amino acid antagonists was undertaken. Using the Warburg manometric technique, it was possible to demonstrate the effectiveness of a specific amino acid antagonist in experiments of short duration. This technique was found to be a desirable supplement to the method of growth measurement as determined in turbidity units by the photoelectric colorimeter. In the latter method a 16-hr. period of incubation in the test medium precedes the turbidity measurements, whereas the Warburg method requires only from 30 min. to 3 hr. per complete experiment. Ely (4), and Greig and Hoogerheide (5, 6), have already successfully applied this technique in the evaluation of germicides.

It was felt that, by a study of bacterial respiration under the influence of metabolite antagonists, it would be possible to gain further information regarding the mechanisms involved in growth inhibition. In this paper the Warburg technique is applied to the problem of elucidating the mechanism of the action of metabolite antagonists in a study of effects of β -2-thienylalanine, β -2-furylalanine, and allylglycine on bacterial respiration of 2 strains of *E. coli*.

¹ This work was supported in part by a research contract with the office of Naval Research.

EXPERIMENTAL

Microorganisms

Two strains of *E. coli* were used for these tests: one listed by the Am. Type Culture Coll. as number 9723, and the other was an unidentified strain designated as Strain N. The test organism was grown in nutrient broth for 18 hr., centrifuged, washed with water, and suspended in the test medium so that 100 ml. contained 3 mg. of bacterial nitrogen.

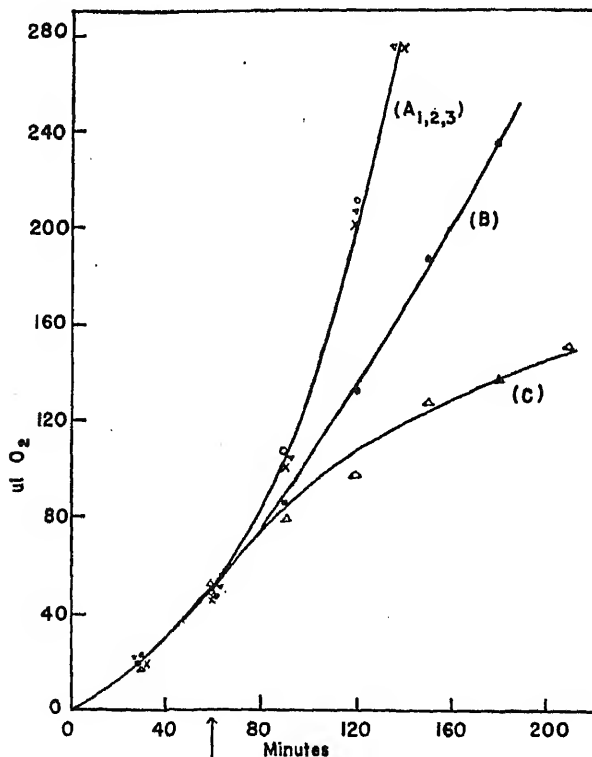


FIG. 1. The effect of β -2-thienylalanine and phenylalanine on oxygen consumption of *E. coli*. Warburg flask contents: 0.25 ml. *E. coli* suspension and 2.75 ml. McLeod's medium. Side arm contents: (A₁) 0.8 ml. H₂O, (A₂) 0.4 ml. thienylalanine (0.02 M), 0.1 ml. phenylalanine (0.02 M), and 0.3 ml. H₂O, (A₃) 0.4 ml. phenylalanine (0.02 M), (B) 0.4 ml. thienylalanine (0.02 M), 0.1 ml. phenylalanine (0.002 M), and 0.3 ml. H₂O, (C) 0.4 ml. thienylalanine (0.02 M), 0.1 ml. phenylalanine (0.0002 M), 0.3 ml. H₂O. Center cup: 0.2 ml. 20% KOH. Arrow indicates point of addition of sidearm contents.

Respiration Measurements

The usual Warburg manometers and flasks were used to measure O_2 consumption. The chamber contained 0.25 ml. of *E. coli* suspension and 2.75 ml. of the medium. The side arm contained a total of 0.8 ml. of antagonist and/or metabolite solution. The center cup contained 0.2 ml. of 20% KOH.

When all solutions were added, the flasks were oxygenated, placed on the manometers, and immersed in the water bath at 38°C. One hr. after equilibrium was reached the side arm contents were emptied into the main flask, and manometric readings were recorded at intervals of 5 min. Bacterial respiration was studied in nutrient broth, in

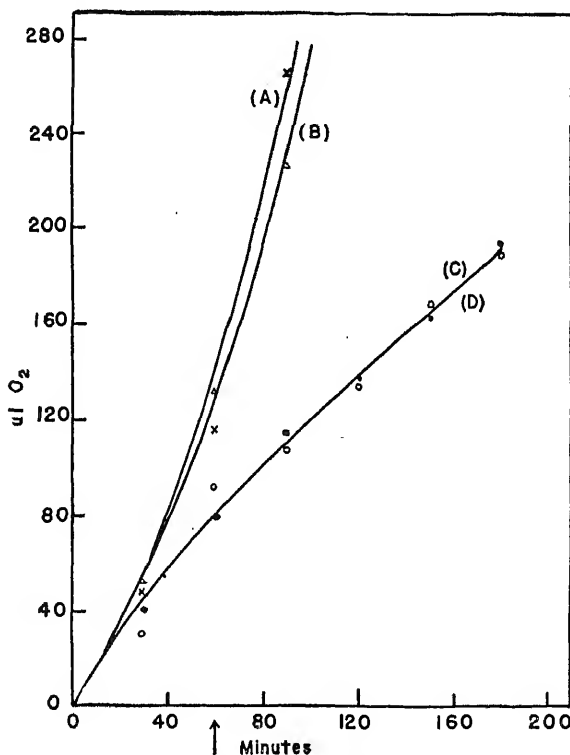


FIG. 2. The effect of β -2-furylalanine and phenylalanine on oxygen consumption of *E. coli*. Warburg flask contents: 0.25 ml. *E. coli* suspension and 2.75 ml. McLeod's medium. Side arm contents: (A₁) 0.8 ml. H_2O —normal control, (A₂) 0.4 ml. phenylalanine (0.02 *M*), 0.4 ml. H_2O , (B) 0.4 ml. furylalanine (0.04 *M*), 0.1 ml. phenylalanine (0.02 *M*) and 0.3 ml. H_2O , (C) 0.4 ml. furylalanine (0.04 *M*), 0.1 ml. phenylalanine (0.002 *M*) and 0.3 ml. H_2O , (D) 0.4 ml. furylalanine (0.04 *M*), 0.1 ml. phenylalanine (0.0002 *M*) and 0.3 ml. H_2O . Center cup: 0.2 ml. 20% KOH.

McLeod's synthetic medium (7), in saline solution, in Ringer's solution, in phosphate buffer solution, and in glucose solution. All solutions were buffered at pH 7.4. The amino acid antagonists, thienylalanine, allylglycine, and furylalanine, and the naturally occurring amino acids phenylalanine and methionine were used in concentrations varying from 0.0001 *M* to 0.2 *M*.

Q_{O_2} determinations of the bacteria were made in the presence of the natural related amino acids alone, the antagonists alone, and the natural amino acids together with their specific antagonists. Although there was some quantitative variation in the Q_{O_2} values, depending on the specific bacterial culture used, the overall results have been

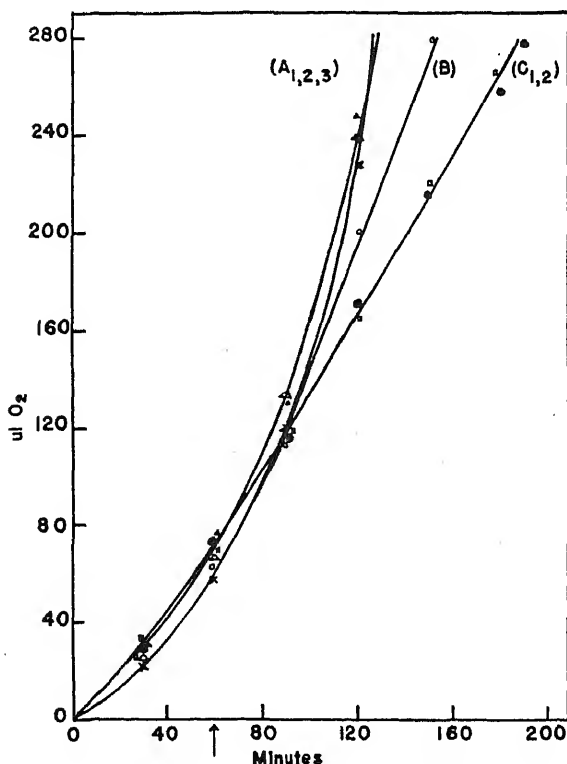


FIG. 3. The effect of allylglycine and methionine on oxygen consumption of *E. coli*. Warburg flask contents: 0.2 ml. *E. coli* suspension and 2.9 ml. McLeod's medium. Side arm contents: (A₁) 0.7 ml. H₂O, (A₂) 0.1 ml. allylglycine (0.1 *M*) and 0.6 ml. methionine (0.1 *M*), (A₃) 0.6 ml. methionine (0.1 *M*) and 0.1 ml. H₂O, (B) 0.1 ml. allylglycine (0.1 *M*) and 0.6 ml. H₂O, (C₁) 0.4 ml. allylglycine (0.1 *M*) and 0.3 ml. methionine (0.1 *M*), (C₂) 0.6 ml. allylglycine (0.1 *M*) and 0.1 ml. H₂O. Center cup: 0.2 ml. 20% KOH.

repeatedly confirmed. The effect of allylglycine on the oxygen consumption of *E. coli* was compared with that on growth as measured in units of turbidity.

Inhibition of Respiration Rate by Amino Acid Antagonists

The curves of Fig. 1 illustrate the distinct inhibition of oxygen consumption of *E. coli* (Strain A.T.C.C. No. 9723) produced by the addition of β -2-thienylalanine. The respiration in all flasks was normal for the first 60 min., after which the antagonist was added to the bacterial suspension from the side arm resulting in a sharply decreased rate of oxygen uptake. The simultaneous addition of phenylalanine prevented the inhibition of oxygen uptake due to thienylalanine.

Figs. 2 and 3 demonstrate the inhibitory action of β -2-furylalanine and allylglycine, respectively.

When bacteria were suspended in media containing constituents inadequate for normal growth, the respiration rate was lower and this respiration was not inhibited by thienylalanine or allylglycine. In fact, the antagonists appear to produce a slight

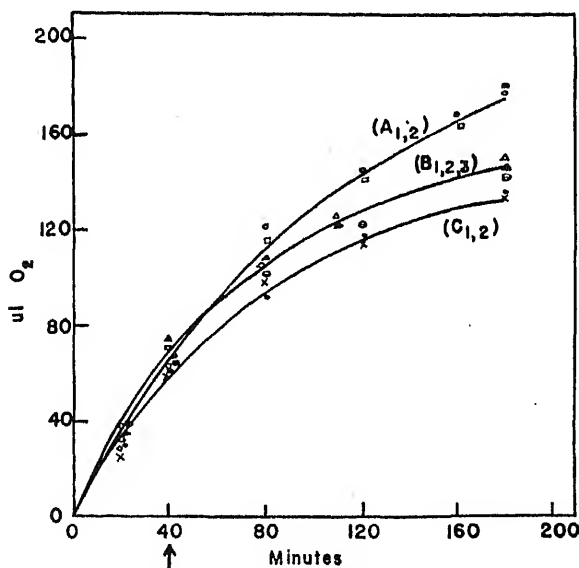


FIG. 4. Lack of inhibitory effect of β -2-thienylalanine and allylglycine on oxygen consumption of *E. coli* in a nonnutritive medium. Warburg flask contents: 2.0 ml. *E. coli* suspension and 1.1 ml. Ringer's solution. Side arm contents: (A₁) 0.6 ml. thienylalanine (0.01 *M*) and 0.1 ml. H₂O, (A₂) 0.6 ml. phenylalanine (0.01 *M*) and 0.1 ml. H₂O (B₁) 0.1 ml. allylglycine (0.1 *M*), 0.4 ml. methionine (0.1 *M*) and 0.2 ml. H₂O, (B₂) 0.6 ml. methionine (0.1 *M*) and 0.1 ml. H₂O, (B₃) 0.1 ml. allylglycine (0.1 *M*) and 0.6 ml. H₂O, (C₁) 0.7 ml. H₂O—normal control, (C₂) 0.6 ml. allylglycine (0.1 *M*) and 0.1 ml. H₂O. Center cup: 0.2 ml. 20% KOH.

stimulation in oxygen consumption when compared to the untreated control culture. These findings are illustrated by the curves in Fig. 4.

To compare the effect of allylglycine on oxygen uptake with that on the growth of *E. coli*, a series of tubes was set up containing the same concentration of *E. coli* as in the Warburg flasks. The tubes contained increasing amounts of allylglycine from none to 30 mg. per tube as indicated in Fig. 5. Turbidity measurements were made at the

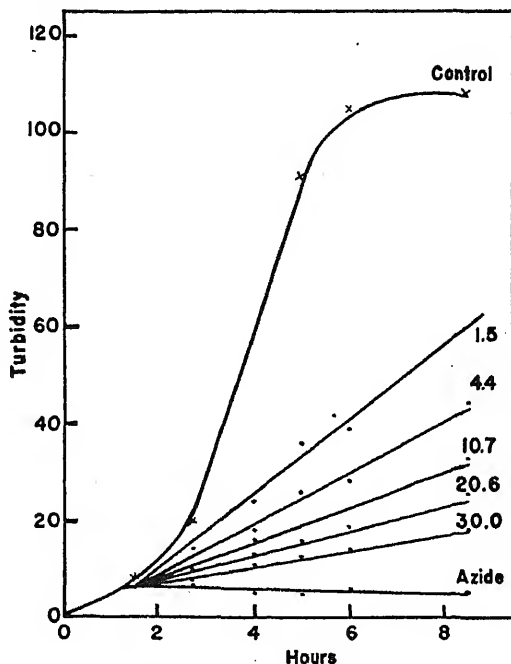


FIG. 5. The growth of *E. coli* under the influence of varying concentrations of allylglycine as measured by the Klett photoelectric colorimeter. Growth is expressed as turbidity in colorimeter units. Allylglycine, expressed in mg., was added to tubes in the amounts shown. Each tube contained 8 ml. of bacterial suspension in McLeod's medium.

end of 1.5, 2.75, 4, 5, 6, and 8.5 hr. When the results of this experiment are compared with the data of Fig. 3, it can be seen that the antagonist-induced inhibition of respiration is parallel to the inhibition of bacterial growth. From these results it is concluded that the decrease in the rate of oxygen consumption is due for the most part to decreased bacterial growth.

It was considered of interest to study the effect on bacterial growth of changing the order of addition of metabolite and antagonist to the bacterial suspension. This experiment is reported in Fig. 6. It is significant that, in the flasks where the antagonist thienylalanine was added after 90 min. to the bacteria-metabolite solution

(B_{1,2}), growth inhibition was significantly less marked than in the flasks where thienylalanine was present initially (C, D). These facts tend to support Barron's views regarding mechanisms of enzyme inhibition, for it may be assumed that, when bacterial cells are initially suspended in a medium containing phenylalanine, certain enzyme surfaces become more or less saturated with the natural amino acid and it is difficult to dislodge these metabolite molecules with the structural antagonist. The reverse of this situation is true when bacterial cells are initially suspended in a medium containing

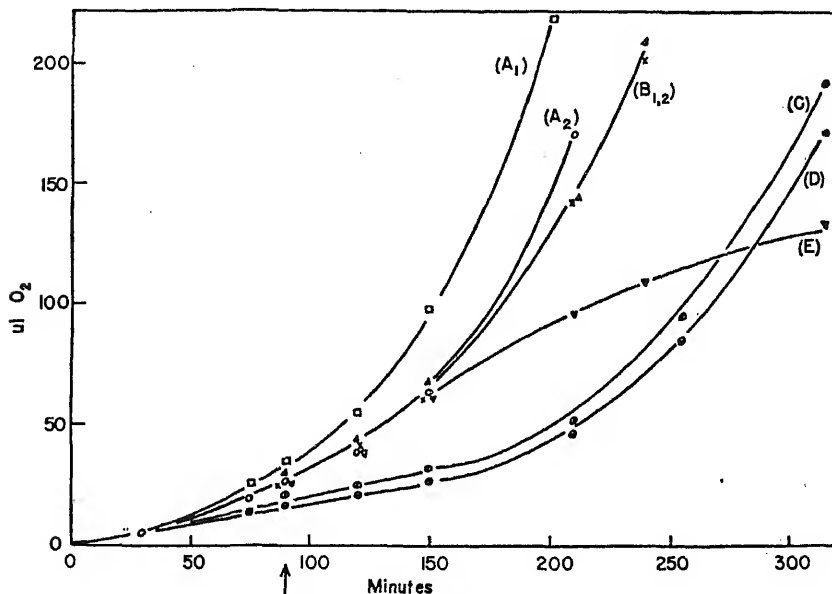


FIG. 6. Effects of changes in the order of addition of metabolite and antagonist on the growth of *E. coli*.

Side arm contents	Warburg flask contents
(A ₁) 0.8 ml. phenylalanine (0.1 M)	0.4 ml. <i>E. coli</i> suspension and 2.8 ml. McLeod's medium
(A ₂) 0.4 ml. H ₂ O—normal control	0.4 ml. <i>E. coli</i> and 3.2 ml. McLeod's medium
(B ₁) 0.6 ml. thienylalanine (0.1 M)	0.4 ml. <i>E. coli</i> , 2.8 ml. McLeod's medium, and 0.2 ml. phenylalanine (0.1 M)
(B ₂) 0.4 ml. thienylalanine (0.1 M)	0.4 ml. <i>E. coli</i> , 2.8 ml. McLeod's medium and 0.4 ml. phenylalanine (0.1 M)
(C) 0.6 ml. phenylalanine (0.1 M)	0.4 ml. <i>E. coli</i> , 2.8 ml. McLeod's medium and 0.2 ml. thienylalanine (0.1 M)
(D) 0.4 ml. phenylalanine (0.1 M)	0.4 ml. <i>E. coli</i> , 2.8 ml. McLeod's medium, and 0.4 ml. thienylalanine (0.1 M)
(E) 0.8 ml. thienylalanine (0.1 M)	0.4 ml. <i>E. coli</i> , 2.8 ml. McLeod's medium

Center cup: 0.2 ml. 20% KOH

thienylalanine. In this case, it may be assumed that enzyme surfaces become saturated with structural antagonist molecules and these are displaced only with difficulty by the natural metabolite.

From the curves of Fig. 1-6 it is evident that the metabolite antagonists studied produce marked growth inhibition which was reversed by the addition of the natural amino acid.

From the data presented, it is concluded that these antagonists act as inhibitors of respiration only by interference with normal bacterial reproduction and are, therefore, effective only in the proliferative stage. In accord with the theories of Fildes (8), Woods (9), and Barron (10), the mechanism of inhibition appears to involve the inactivation of a vital metabolite necessary for growth perhaps by blocking an active center in a bacterial enzyme system. In an effort to gain information regarding the mechanism of this inactivation a study of the effect of metabolite antagonists on a lactic dehydrogenase system was undertaken. Results of this study indicate that this dehydrogenase system is not involved in the mechanism of growth inhibition. Further work on the detailed mechanism of this inactivation involved a study of the effects of metabolite antagonists on an *E. coli* lactic dehydrogenase system. This system was not inactivated by thienylalanine, furylalanine or allylglycine.

SUMMARY

A study of the effects of specific amino acid antagonists on the respiration of two strains of *E. coli* is reported, using the Warburg manometric technique.

The amino acid antagonists, β -2-thienylalanine, β -2-furylalanine, and allylglycine, were found to markedly inhibit the rate of oxygen consumption of *E. coli* by inhibiting growth.

The inhibition was nullified by the simultaneous addition of the natural amino acids phenylalanine and methionine respectively. No inhibition of respiration was noted when the organism was cultured in a medium inadequate for growth.

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The Oxidative Inactivation of Poison Ivy Allergens and Related Products by Laccase ¹

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INTRODUCTION

In view of the demonstration by Sizer and Prokesch (1,2), and confirmation by Mason *et al.* (3), that poison ivy and related skin irritants can be oxidatively inactivated by tyrosinase, it seemed likely that other enzymes which oxidize phenolic derivatives might also attack the poison ivy allergens. The most promising of these oxidases appeared to be laccase, since it, like tyrosinase, is a copper protein (4) and attacks many of the same substrates. The specificities of the two enzymes are by no means identical, however, since laccase has a very much greater relative activity toward *o*-cresol, hydroquinone, *p*-phenylenediamine and guaiacol, but is appreciably less active than tyrosinase toward tyrosine and *p*-cresol (5-8).

Since its discovery by Yoshida in 1883 (9) in the sap of the Japanese lac trees, *Rhus succedanea* and *Rhus vernicifera*, laccase has been considered responsible for the conversion of the yellow sap to solid black lac, presumably as a result of the oxidation of phenols which are present, especially urushiol, the major allergen of poison ivy (10,11,12). Since the poison ivy plant, *Rhus toxicodendron*, belongs to the same family as the lac trees, it seemed possible that a laccase might be isolated from the poison ivy plant which would oxidize and inactivate the allergens produced by this plant as well as the active components in lac latex (12,13). Similarly, laccase of the lac tree might be expected to have an effect on the allergens of the poison ivy plant. McNair in 1917 (13) prepared an oxidative enzyme from *Rhus diversiloba* (poison oak) which causes the sap of this plant to darken to a black shiny varnish-like substance. Accompanying this darkening, a decided loss of toxicity of the sap to human skin was demonstrated. Experiments by McNair (14) with this enzyme as a therapeutic agent in the treatment of poison ivy dermatitis were unsuccessful, however, although the poison ivy oxidase did not irritate the epithelium.

¹ Aided by a grant from Eli Lilly and Co.

EXPERIMENTAL

Laccase Preparations

All poison ivy laccase preparations were made from pulverized poison ivy leaves extracted with 95% alcohol.² In preliminary experiments this powder was used as the laccase preparation, but since it contained small residual amounts of poison ivy allergens, it was necessary to purify the laccase further. The skin irritants could be removed by extracting 6 times with ether. In the purification of laccase a 10% aqueous extract of the powder was prepared. The laccase was then precipitated by the addition of 2 volumes of *M*/20 phosphate buffer, pH 7.3. This laccase preparation was very active, although it underwent a slow autoxidation on standing.

The laccase from the Japanese lac tree was prepared from the crude, partially oxidized latex (kiurushi³). This contains 70% lacquer substances (urushiol and other allergens) as well as 20% water and 10% gums and nitrogenous substances (15). To free the lac of materials soluble in organic solvents, the crude kiurushi is extracted successively with 95% alcohol, then ether, followed by acetone. The residue, freed of fat-soluble substances, is further purified by dissolving in water and precipitating with 2 volumes of 95% alcohol. The precipitate is then extracted several times with alcohol and ether before use. The above procedure is essentially that of Suminokura (15). Several laccase preparations were further purified according to Keilin and Mann (16) by dissolving the above precipitate in a small amount of water and precipitating at pH 4.95 with saturated ammonium sulfate. The solution was centrifuged, the centrifugate taken up in water, and dialyzed for 18 hr. against running tap water. This preparation was then lyophilized to yield a fluffy white powder which had good laccase activity when suspended in water.

The activities of both the laccase from poison ivy and that from Japanese lac were tested with hydroquinone, *p*-cresol, *o*-cresol, catechol, *p*-phenylenediamine, L-tyrosine, pyrogallol, guaiacol, tyramine, and vanillin. The laccases from these 2 different species of *Rhus* were found to be very similar in their specificities and general properties. The characteristic differences between laccase and tyrosinase reported by others (5-8) were also found for these preparations.

Manometer Studies

The Barcroft differential respirometer was used to measure oxygen consumption at 37°C. at a shaking rate of 85 strokes/min. Single side-arm flasks of 7 ± 0.1 ml. capacity were used throughout for both control and experimental solutions. It was found unnecessary to take into account the slight differences between the oxygen constants of the control and reaction vessels in the calculations of oxygen consumption. In a typical experiment 0.15 ml. 10% commercial poison ivy extract in acetone was placed in the cup. The acetone was then evaporated leaving a film of poison ivy allergens in the bottom of the flask. Then 1.5 ml. *M*/20 phosphate buffer, pH 7.3, were added, and

² Kindly furnished by Dr. Walter Christiansen of E. R. Squibb and Sons.

³ The kiurushi and pure urushiol were kindly furnished by Dr. Merrill W. Chase, Rockefeller Institute for Medical Research.

0.15 ml. laccase solution placed in the side-arm. After temperature equilibration the enzyme was tipped into the main chamber. The control flask was identical in all respects, except that the laccase was not mixed with the poison ivy allergens. By such an experimental arrangement any autooxidation of either the poison ivy preparation or of the laccase was corrected for, so that the manometer registered only the oxygen consumption of the toxicant catalyzed by laccase. While this experimental setup is perhaps the best available, it is not ideal, since autooxidation may not be quite identical in the two flasks. Autooxidation has been studied quantitatively for these poison ivy allergens by Sizer and Prokesch (2) who found that the autooxidation of these compounds proceeded relatively slowly as compared with oxidation by tyrosinase. The same is true, when autooxidation is compared with oxidation by laccase. A modified technique was frequently used in the laccase experiments. Instead of using unmixed substrate and enzyme in the control flask, the laccase was inactivated by boiling. In other experiments enzyme was omitted from the control flask. Results obtained by these 3 techniques were very similar. In preliminary studies no carbon dioxide evolution could be demonstrated; hence, in subsequent experiments alkali was omitted from the central well of the reaction vessels.

Typical results, showing the action of poison ivy laccase in catalyzing the oxidation of typical crude and purified poison ivy allergen preparations, are shown in Fig. 1. The data are very similar when Japanese lac

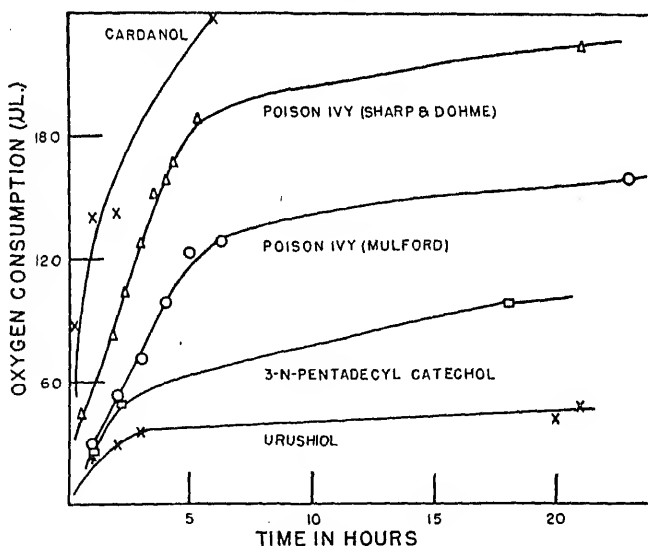


FIG. 1. The oxidation by poison ivy laccase of crude extracts and purified poison ivy allergens. The experimental Barcroft manometer vessel contained 0.15 ml. laccase solution, 0.1 ml. (2-10 mg.) allergen (solvent evaporated off) and 1.5 ml. *M*/20 phosphate buffer, pH 7.3. The control flask was the same, but with buffer solution instead of laccase.

tree laccase is used. Rates of oxygen consumption of each allergen catalyzed by each laccase were calculated from data obtained during the first few hours of the reactions, and are summarized in Table I. From inspection of the figure and the table, it is apparent that both laccases cause the rapid oxidation of the various poison ivy crude and purified allergens, although the rate varies greatly for different preparations. The two laccases, although similar, are by no means identi-

TABLE I
The action of Laccase on Poison Ivy Allergens as Measured by Oxygen Consumption, Increase in Color, and Decrease in Dermatitis-Producing Properties

Poison ivy allergen ^a	Oxygen consumption (μl./hr.)		Color change ^b		Toxicity ^c	
	Ivy leaf laccase	Lac latex laccase	Ivy leaf laccase	Lac latex laccase	Ivy leaf laccase	Lac latex laccase
3- <i>n</i> -pentadecyl catechol	29	7	Purple	Purple	Decrease	Decrease
Anacardol	26	42	Brown	Gray	Decrease	Decrease
Anacardic acid	6	78	Brown	Yellow	Decrease	Decrease
Urushiol	24	60	Green	Brown	Decrease	Decrease
Mulford poison ivy	29	54	Brown	Brown	Decrease	Decrease
Sharp & Dohme poison ivy	47	181	Green	Green-brown	Decrease	Decrease
Reichel poison ivy	47	240	Green	Brown	Decrease	Decrease
Wyeth-Reichel poison ivy	34	210	Yellow-green	Brown	Decrease	Decrease
Squibb poison ivy	57	240	Yellow	Brown	Decrease	Decrease
Japanese lac	12	26	Brown	Brown	Decrease	Decrease
Cashew nut shell extract	8	—	Brown	—	Decrease	—
Cardanol	21	—	Brown	—	—	—

^a The allergens were kindly furnished as follows: 3-*n*-pentadecyl catechol, Dr. Howard Mason; anacardol and anacardic acid, T. E. Knock, Armour Research Foundation; urushiol and Japanese lac, Dr. Merrill W. Chase; Mulford poison ivy, Mulford Colloid Laboratories; Reichel and Wyeth-Reichel poison ivy, Wyeth, Inc.; Squibb poison ivy, E. R. Squibb and Sons; cashew nut shell extract and cardanol, Irvington Varnish and Insulator Company.

^b A color of much lower intensity also developed in the control solution lacking laccase.

^c The dermatitis-producing properties of the laccase-treated allergens were tested on guinea pig and human skin. If the dermatitis of the experimental area is less than that of the control, it is listed in the table as a decrease in toxicity.

cal; the lac enzyme is somewhat more effective on most substrates than the ivy laccase. It is interesting to note that the poison ivy enzyme oxidizes not only the allergens of poison ivy, but also the components of Japanese lac (kiurushi). Similarly, laccase of the lac tree oxidizes toxicants from both sources.

Since the reacting systems are grossly heterogeneous, because of the insolubility of the poison ivy allergens, it seemed possible that the addition of an emulsifying agent might have the effect of speeding the enzyme reaction. This was studied by adding 0.01 ml. of the synthetic detergent "Tween-20" to the reaction flasks. Under these conditions an emulsion was formed in which the enzymic oxidation was found to proceed somewhat more rapidly than in the absence of the detergent.

Spectroscopy Studies

During the course of the oxidation of the poison ivy allergens by laccase the solution gradually assumed a darker color as compared with

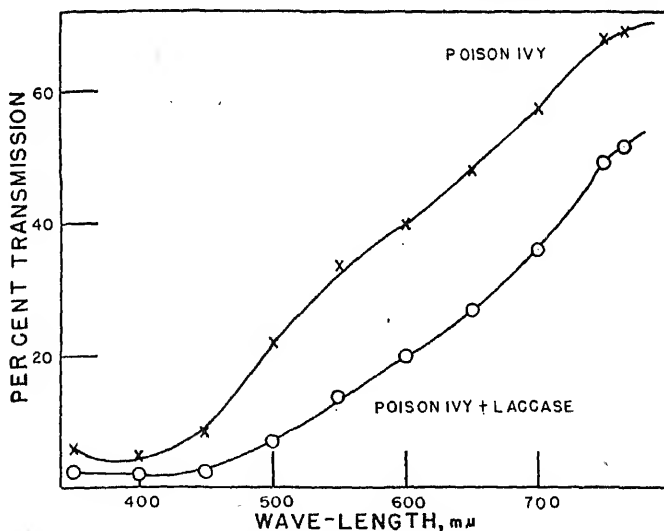


FIG. 2. The effect of Japanese lac tree laccase on the visible absorption spectrum of Mulford extract of poison ivy allergens. The experimental tube contained 0.3 ml Mulford poison ivy, 0.3 ml. (5 mg.) purified laccase solution and 4.6 ml. *M*/20 phosphate buffer, pH 7.3. The control tube was identical, except that the laccase had been inactivated by boiling. The oxidation proceeded for 2 hr. at 37°C. before the absorption curves were made.

the control, which also changed color to a lesser degree due to autoxidation. This is recorded qualitatively in Table I, from which it appears that the actual color produced varies for different substrates. To study this increase in color more quantitatively the absorption in the visible region of the spectrum was studied using the Coleman Universal Spectrophotometer. In Fig. 2 is shown the absorption of Mulford poison ivy extract and of Mulford poison ivy extract oxidized by lac tree laccase. Both preparations had been incubated for 3 hr. at 37°C. at pH 7.3. It is clear from the figure that laccase has caused an increase in absorption of the poison ivy allergens throughout the visible spectrum. Studies using the Beckman Spectrophotometer indicate that this effect on the absorption spectrum continues through the ultraviolet region as well.

It is possible to utilize this change in color of poison ivy allergens by laccase as a means of studying the kinetics of the reaction, just as oxygen consumption was used to follow the course of the oxidation.

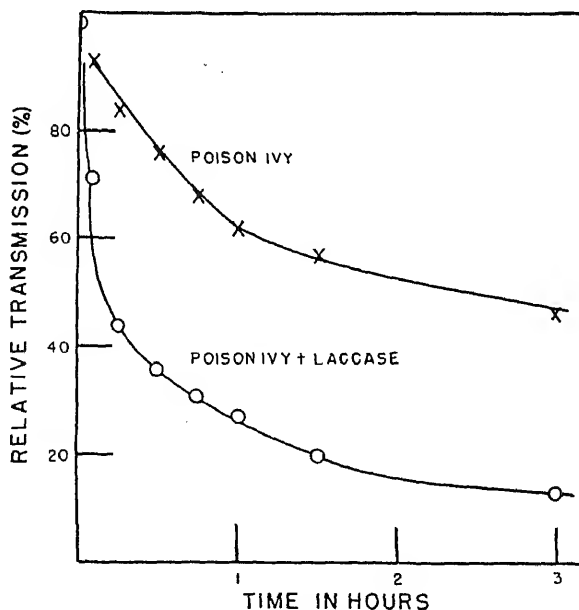


FIG. 3. The effect of lac tree laccase on Mulford poison ivy allergens as measured by the change in relative per cent transmission of the solution as a function of reaction time. Experimental and control solutions are identical with those used for Fig. 2.

Such a study at 600 m μ , using the same system as in Fig. 2, is presented in Fig. 3. The control undergoes some autoxidation, but changes color much more slowly than does the experimental, in which there is an oxidation of the poison ivy toxicants by laccase. In this experiment the oxidation occurs more rapidly at first, and is practically completed in 1.5 hr. In general, however (except for the Mulford preparation, which is an aqueous colloid), this spectroscopic technique for studying the kinetics is not very satisfactory, because most of the poison ivy preparations do not yield homogeneous aqueous solutions transparent enough for study. It is, therefore, usually necessary to extract the solutions with ether at the end of the oxidation by laccase and then study the absorption of the ether solutions. An alternative method is to stabilize the emulsion with a detergent inert to enzymes such as Tween 20.

The increase in absorption of poison ivy allergens after treatment with laccase is very similar to the results obtained with tyrosinase.⁴ Similar shifts in absorption were obtained by Mason (3) after oxidation of catechol and related compounds by tyrosinase. In all cases it seems probable that the changes in absorption reflect the conversion of phenolic to quinone groups followed by rearrangements in the molecule and polymerization to colored end products (17).

Studies on Biological Activity

Since the action of laccase on poison ivy toxicants parallels closely that of tyrosinase with reference to oxygen consumption and change in absorption spectrum, it seemed possible that laccase might also resemble tyrosinase in causing a decrease in the ability of the allergens to produce dermatitis (2).

The technique used was the same as that of Sizer and Prokesch (2). Both guinea pigs and humans were used as test animals; the guinea pig abdomen was shaved 2 days before the test. When humans were used the skin of the forearm or leg was prepared by washing with soap and water, and the hair was usually removed with an electric shaver. Before applying the poison ivy allergen to the skin it was taken up in ether. A glass ring 1.75 cm. in diameter and 0.5 cm. high was held firmly against the skin. An aliquot (usually 0.1 ml.) of the ether extract was then spread on the area of skin inside the ring. The ring was removed after the solvent had evaporated. To avoid spreading by contact with clothing the human areas were covered with "band-aids," while the guinea pig areas were left uncovered. The allergen was left on the skin for

⁴ Unpublished data.

24 hr., or until dermatitis was apparent, at which time the residual allergen was removed with soap solution or alcohol and ether. Experimental and control areas were always treated identically. Attempts were made to adjust the dosage used to just above the threshold level in order to avoid a severe dermatitis, and since, at this level, differences between control and experimental dermatitis areas are most apparent.

The diagnosis of the comparative dermatitis of the control and experimental areas was always made by at least 2 observers. Appraisal of the severity of the dermatitis was made on the basis of the degree of edema and erythema and (in humans only) intensity of pruritus. The diagnosis was usually repeated on 3 successive days. A decision was then made as to whether the dermatitis of the experimental area (poison ivy allergen previously oxidized with laccase) is more or less severe than the control area (allergen not treated with laccase). The results of the skin tests are summarized in Table I, from which it can be seen that, in all cases, the previous oxidation of the allergens with laccase has resulted in an appreciable decrease in the dermatitis-producing properties of the poison ivy preparation. In most experiments, however, the laccase only partially destroyed the irritant properties of the allergen.

A variation of the above technique for measuring the effect of laccase upon the biological activity of the poison ivy allergens involved the use of laccase as a prophylactic agent. On the experimental area of the skin was placed 0.3 ml. of laccase, while the control area was coated with boiled (inactive) laccase. The solutions were evaporated to dryness before the addition to each area of 0.1 ml. poison ivy preparation. In most experiments, the dermatitis which developed was appreciably less severe on the area previously treated with active enzyme than on the control which was coated with the boiled enzyme. The reverse experiment ("curative" rather than "prophylactic" technique), where the allergen was placed on the skin several hours before the enzyme was added, was relatively unsuccessful in most cases, possibly because the allergen penetrated into the skin before the allergen could be oxidized by the laccase, to which the skin is relatively impermeable. These results with laccase as a therapeutic agent are quite similar to those obtained by McNair (14).

DISCUSSION

In view of the general similarity in properties of laccase and tyrosinase, it is not surprising to find that poison ivy allergens are oxidized by both enzymes. The types of oxidation induced by these two enzymes are doubtless quite comparable, since oxygen consumption, change in visible and ultraviolet absorption, and decrease in irritant properties of the allergens, are much the same when induced by the 2 enzymes. The similarity in general properties of laccase from Japanese lac and from poison ivy leaves is consistent with the fact that these laccases come from the same family of plants, which produce the same types of skin irritants. It seems very likely, for the various species of the *Rhus* family, that laccase may be concerned with the metabolism of these phenolic allergens.

Since both tyrosinase and laccase contain about 0.2% copper (12), it is probable that the metal is closely concerned with the catalytic action of these enzymes, and might have some effect by itself on poison ivy allergens. Several experiments have been run to test this possibility. When 0.2 mg. powdered Cu was added to 0.1 ml. allergen preparation in phosphate buffer, pH 7.3 at 37°C., a positive effect was apparent as shown by oxygen consumption, increased absorption in the visible and in the ultraviolet, and a decrease in the dermatitis-producing properties of the allergen. A number of phenolic derivatives such as catechol, 3-*n*-pentadecyl catechol, crude Japanese lac, and several different commercial poison ivy preparations, were oxidized by the powdered copper. These experiments illustrate the fact that copper, although much less efficient than the copper enzymes, can oxidize the poison ivy allergens in a manner similar to the action of tyrosinase and laccase.

ACKNOWLEDGMENT

It is a pleasure to acknowledge the technical assistance of Mrs. Elma Logemann in this study.

SUMMARY

Laccase preparations have been made from poison ivy leaves and from the latex of the Japanese lac tree. Laccases from these sources were very similar and showed the characteristic similarities and differences when compared with tyrosinase. Like tyrosinase, the laccases were found to oxidatively inactivate certain poison ivy allergens; these included the purified compounds 3-*n*-pentadecyl catechol, anacardol, anacardic acid, and urushiol, as well as many crude commercial poison ivy preparations, and the allergens of Japanese lac latex. The conclusions on the oxidation and inactivation of these allergens by laccase were based on manometric measurements of an increase in oxygen consumption, spectrophotometric measurements of an increase in absorption in the visible and ultraviolet spectrum; and measurements of the decrease in biological activity in skin tests on guinea pigs and humans. To a lesser degree, this oxidative action of copper enzymes on poison ivy allergens is shared by inorganic copper.

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Choline—The Cause of Lipocaic Lipotropic Action on Fatty Rat Livers

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INTRODUCTION

In 1936, Dragstedt and his associates (1,2) reported that a pancreatic tissue extract prevented fatty infiltration of the liver in depancreatized dogs. It was believed that the amount needed to demonstrate its lipotropic activity was much smaller than would be needed on the basis of its choline content. This was interpreted as evidence for the existence of a lipotropic factor other than choline, and the unidentified material was called "lipocaic." Although the above work was carried out on depancreatized dogs, numerous workers have employed rats maintained on a high fat-low protein diet for testing the efficacy of lipocaic (3). Best and Ridout (4) and Aylward and Holt (5) reported that lipocaic had no activity in rats other than that which could be accounted for on the basis of its content of choline and protein. MacKay and Barnes (6) suggested that the choline and protein content of lipocaic must be considered before assuming the presence in lipocaic of a fat-metabolizing factor. In contrast to these findings, Channon, Loach and Tristram (7) found that choline could only account for one-third of the activity of lipocaic and that the non-choline activity is not accounted for by the protein content of the extracts. In more recent work, Dragstedt and coworkers (8,9) have reported that the lipotropic effect of lipocaic on dietary fatty livers in rats must be due to some constituent other than choline, methionine, inositol or the non-specific action of protein. In view of the above conflicting results, we have reinvestigated the effects of lipocaic and choline on the fatty livers of rats receiving high fat-low protein diets.

EXPERIMENTAL

Preparation and Analyses of Pancreas Extract

The pancreas extract was prepared with the recent modifications listed by Dragstedt *et al.* (8). The glands were obtained frozen from the packing house, immediately ground in the frozen state with a meat grinder, and dropped with stirring into acidified 95% ethyl alcohol. Two lots of lipocaic were prepared, starting with 60 and 30 lb. quantities of the pancreas gland. Lots 1 and 2 gave yields of 194 and 143 mg., res-

TABLE I
Microbiological Analyses of Lipocaic

Analysis as indicated	Per cent found
Arginine	1.37 <i>L. casei</i>
Aspartic acid	3.34 <i>L. mesenteroides</i>
Cysteine (1) (or cystine)	0.28 <i>L. mesenteroides</i> . Not corrected for loss on acid hydrolysis
Glutamic acid (2)	8.1 <i>L. arabinosus</i>
Glutamic acid (3)	6.72 <i>S. faecalis</i>
Histidine	1.14 <i>L. mesenteroides</i>
Isoleucine	3.50 <i>L. arabinosus</i>
Leucine	5.80 <i>L. arabinosus</i>
Lysine	5.60 <i>L. mesenteroides</i>
Methionine (4)	1.45 <i>L. fermenti</i>
Methionine (5)	1.39 <i>L. mesenteroides</i>
Phenylalanine	2.65 <i>L. mesenteroides</i>
Serine (7)	1.13 <i>L. mesenteroides</i> . Unpublished method
Tyrosine (8)	0.78 <i>L. casei</i> . Alkaline hydrolysis. May be low due to incomplete racemization
Tryptophan (9)	0.87 <i>L. arabinosus</i>
Valine	4.54 <i>L. arabinosus</i>
Glycine (10)	6. One significant figure only
Choline (11)	3.9 <i>Neurospora</i>
Inositol (12)	1.1 Yeast growth
Threonine	2.72 <i>L. fermenti</i>
Nitrogen	11.62

Notes 2 and 3. *L. arabinosus* is not specific for L-(+)-glutamic acid. The value 8.1 may include some unnatural glutamic acid since the *S. faecalis* value, which is specific, is lower. This was not further pursued.

Notes 4 and 5. *L. fermenti* responds equally to both D- and L- methionine. *L. mesenteroides* responds only to L-methionine. Nothing is known of their response to choline.

pectively, of lipocaic/100 g. of fresh pancreas which is comparable to that reported by Dragstedt. The lipocaic (lot 1) was subjected to microbiological analysis.¹ The results

¹ The microbiological analyses were carried out by Dr. S. Shankman of the Shankman Laboratories, 2023 So. Santa Fe Avenue, Los Angeles.

are given in Table I. Over 65% of the total solids in the extract can be accounted for by the 18 analyzed compounds. The chemical analysis for choline when carried out by the method described by Glick (10) gave a value of 4.4%. This agrees well with the 3.9% obtained by the microbiological method. Dragstedt and coworkers (8) reported that no choline was found in their preparation of lipocaic.

Method of Assay

White male rats ranging from 120 to 150 g. were used. The animals were removed from the stock diet (Purina Dog Chow) and placed on wire racks with 6 rats per group. The basal diet consisted of 5% vitamin test casein (General Biochemicals, Inc.), 5% Brewers' Yeast (strain G—Anheuser-Busch, Inc.), 2% "Celluration" (Fisher Sci. Co.), 5% salt mixture (No. 1 Harris Laboratories), 42% glucose, 40% butter fat, and 1% cod liver oil. The supplements in each case were kneaded into the basal diet which was fed *ad libitum*. The lipocaic was added at the 2% level, since preliminary experiments indicated that this amount would produce a substantial lowering of liver fat. In the other groups, the supplements were added in the concentration and ratio found by the microbiological analyses and on the basis of adding 2% lipocaic to the basal diet. For example, the analyses gave a value of 3.9% for choline; thus, the equivalent amount of choline in the diet containing 2% lipocaic is 78 mg. of choline/100 g. of diet. The rats were maintained on the experimental diets for 20 days, after which time they were etherized and then bled to death to free the body of excess blood. The livers and eviscerated bodies were digested separately with hot alcoholic KOH. The liver and body fats were determined by petroleum ether extraction of the acidified material and therefore represent fatty acids plus unsaponifiable material.

RESULTS

Average results comprise Table II. Lipocaic when incorporated into the basal diet at the 2% level has a marked lipotropic effect. This lowering of liver fats can be attributed to its choline content as evidenced by the comparable results obtained in Gp. 3, 4, and 5 in Exp. 1 and Gp. 3 and 4 in Exp. 2. Interestingly enough the decrease in liver fat in the supplement-fed groups resulted in an increase in total body fat. In Exp. 1 this can be partly attributed to the increased food consumption of Gp. 2, 3, 4, and 5. In Exp. 2, the food consumption for all groups was constant. This was accomplished by limiting the available food to Gp. 2, 3, and 4 to that eaten voluntarily by Gp. 1 receiving the basal diet. In this case Gp. 2, 3, and 4 were started on their experimental diets 24 hr. later. The results in Exp. 2 again show the lipotropic action of the pancreas extract, and that its activity can be accounted for by its choline content. Other experiments, not reported here, were carried out

TABLE II

The Effect of Lipocaic, Choline and Amino Acids on Liver and Body Fat

Exp.	Group ^a	Test substance	mg./ rat/day	Average initial body wt.	Average wt. gain	Food intake	Wt. liver	Total fat in liver	Liver fat	Body fat
				g.	g.	g./rat/ day	g.	g.	per cent	per cent
I	1	Basal diet	—	137	-5.8	6.8	6.3	1.22	19.4	10.7
	2	Lipocaic No. 1 (2%)	162	137	+5.5	8.1	5.4	0.45	8.3	14.5
	3	Amino acid mixture plus choline	85	137	+9.6	8.0	5.8	0.50	8.6	15.0
	4	Choline (0.078%)	5.3	137	+4.1	6.8	5.2	0.45	8.6	12.7
	5	Choline (0.078%) plus methionine(0.029%)	5.7 2.1	137	+3.0	7.4	5.5	0.41	7.5	12.1
II	1	Basal diet	—	135	-11.5	6.3	5.9	1.02	17.3	9.3
	2	Lipocaic No. 2 (2%)	126	135	-12.5	6.3	3.9	0.20	5.0	11.1
	3	Choline (0.078%)	4.9	135	-13.1	6.3	3.8	0.27	7.2	9.6
	4	Choline (0.078%) plus methionine(0.029%)	4.9 1.8	136	-6.4	6.3	4.2	0.32	7.6	10.7

^a 6 male rats in each gp.

to determine the comparative potency of the pancreas extracts—lots 1 and 2. Both lots were comparable in choline content and ability to prevent fatty infiltration of liver.

Entenman and Chaikoff (11,12) have demonstrated the presence in the pancreas of a substance other than choline that prevents fatty livers in completely depancreatized dogs maintained with insulin. This material is extracted from the gland with dilute acid and is further purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation. The relationship between lipocaic and the antifatty liver factor described by Entenman and Chaikoff is not known at the present time.

Although the evidence presented here indicates that the lipotropic activity of lipocaic in the dietary fatty liver of rats on a high fat-low protein diet can be accounted for by its choline content, it is necessary to emphasize, in agreement with Entenman and Chaikoff (11), that the mechanisms for the production and cure of fatty livers in the depancreatized dogs and normal rats have not been shown to be identical and results obtained from one type of experimental animal cannot be applied to the other.

SUMMARY

The lipotropic action of *lipocaic* on the fatty livers of albino rats receiving a diet low in protein and high in fat was found to be due to the choline content of this preparation.

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Preparation of *p*-Dimethylaminoazobenzene Containing Isotopic Nitrogen

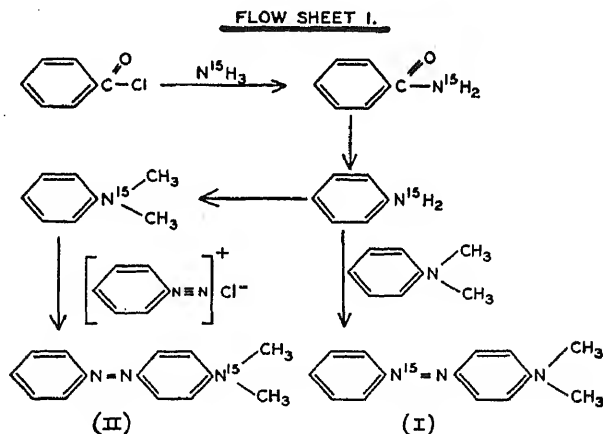
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INTRODUCTION

p-Dimethylaminoazobenzene (hereafter called DAB), containing N^{15} as a tracer element in each of the 3 possible positions, has been prepared so that a study of the metabolism of this well known hepatic carcinogen may be made.



DAB containing N^{15} in the aniline residue (I), or in the dimethylamino group of the *p*-aminodimethylaniline moiety (II), were prepared using the reactions shown in Flow Sheet 1. Allen and Wilson (1) reported the preparation of isotopic aniline by essentially the method shown in the Flow Sheet but stated the yields were unsatisfactory.¹

¹ Preliminary experiments using sodium hypobromite gave a 60-70% yield of aniline.

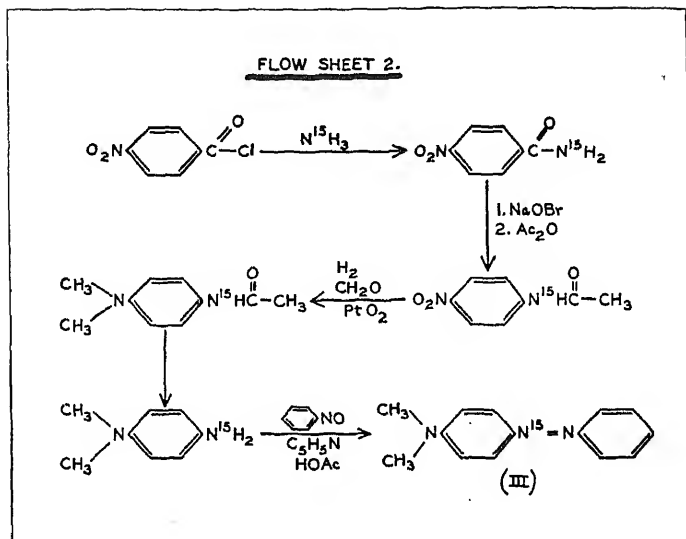
However, by substituting sodium hypochlorite for the bromite, consistent yields of 85–91% were obtainable and by this method isotopic aniline was prepared in 88% yield based on the ammonia used.

The isotopic aniline was methylated in 78% yield by a modification of the method Birkofer (2) used for preparing dibenzylaniline. Isotopic aniline and isotopic dimethylaniline were coupled with the appropriate amine to yield I (95.5%) and II (85%), respectively. The coupling reaction was carried out essentially as described by Clarke and Kirner (3) for the preparation of methyl red. The higher yield of I than of II was undoubtedly due to the fact that, in the preparation of I, the reaction could be driven to completion by the addition of excess non-isotopic dimethylaniline, whereas such was not practical in the case of II. Similar yields were obtained in the model experiments where purified materials were used.

Cleavage of DAB (I) (N^{15} at.-% excess 2.0, 2.1) by hydrogenation, isolation of the resulting amines, and mass spectrographic analysis of the products showed the N^{15} at.-% excess in the aniline residue to be 5.2 and in the *p*-aminodimethylaniline to be 0.87. Since the amines were not separated in the pure state, these data, together with the method of synthesis, were considered to show that the N^{15} was in the aniline residue of DAB (I).

Considerably more difficulty was encountered in obtaining the DAB (III) with isotopic nitrogen in the amino group of the *p*-aminodimethylaniline residue. Preliminary experiments showed it was feasible to condense *p*-aminodimethylaniline and nitrosobenzene to obtain DAB by following the procedure Ruggli and Stauble (4) used for condensing sulfanilic acid and nitrosobenzene. Thus the problem became one of preparing *p*-aminodimethylaniline labeled in the required position. It was hoped that this could be accomplished by the Hofmann degradation of *p*-dimethylaminobenzamide containing isotopic nitrogen in the amide group. However, in preliminary experiments, the action of sodium hypochlorite in either alcoholic or aqueous solution led only to the recovery of unreacted amide and the corresponding acid. This method was then abandoned and the desired amine was obtained by the series of reactions shown in Flow Sheet 2.

p-Nitrobenzamide containing N^{15} was prepared quantitatively in the usual manner and this was converted to the amine, by a modification of the method of Hauser and Renfrow (5), in a yield of 75% based on amide used, and 89% based on unrecovered ammonia. The crude



nitroaniline was purified by formation of the corresponding acetanilide (6).

Reductive methylation of the nitroacetanilide, after the manner of Emerson and Uraneck (7), led to the desired *N*-acetyl-*p*-aminodimethylaniline in 80% yield. Hydrolysis with dilute HCl yielded 92% of the desired amine, which was then coupled (4) with nitrosobenzene to give DAB (III) in 80% yield.

The isotopic nitrogen was shown to be in the desired position in *p*-aminodimethylaniline by diazotization, decomposition of the diazonium salt with alkali, and isotopic analysis of the evolved nitrogen. The isotope was thus found to be exclusively in the primary amino group.

EXPERIMENTAL ^{2,3,4}

Benzamide

In a 3-necked round bottom flask equipped with a stirrer, an inlet tube and an outlet to a boric acid trap for any unreacted ammonia, there was placed 12 g. of benzoyl chloride and 250 ml. of ether. The solution was cooled in a dry ice-acetone

² Microanalyses by R. J. Koegel.

³ N^{15} ratios by B. W. Burr using a Consolidated mass spectrometer. No. 21-102.

⁴ All m.p.'s uncorrected unless otherwise indicated.

bath and, while being stirred, there was passed in the ammonia generated by refluxing a solution of 8.21 g. of NH_4Cl (N^{15} at.-% excess 61.5) in 100 ml. of 20% NaOH solution. Four hr. were necessary to carry out the reaction, and then the mixture was allowed to stand at room temperature overnight.

The ether was removed by filtration and the solid washed with five 50 ml. portions of absolute ethanol. The ether was distilled from the combined filtrate and ethanol washings, after which the residue was concentrated to incipient crystallization and 200 ml. of benzene was added. The solution was heated to boiling, filtered, and the residue washed 3 times with 100 ml. portions of hot benzene. This residue, plus that from the alcohol washings, amounted to 3.95 g. (96.5%) of recovered ammonium chloride.

The benzene-alcohol solution was concentrated to 100 ml. and, after cooling, the crystals were collected by filtration. Further concentration to 20 ml. gave another crop of crystals which brought the total yield to 9.0 g. (97.5%) of benzamide, m.p. 121–123°C. (N^{15} at.-% excess. Calc'd: 61.5; found: 60.6.)

Aniline (1)

Into a solution of 30 g. of NaOH in 100 ml. of water and 150 g. of ice there was introduced 8.2 g. of chlorine generated by the action of excess concentrated HCl on 7.8 g. of KMnO_4 . To the cold stirred solution there was added 12.4 g. of benzamide; the reaction mixture was heated quickly until refluxing began, and kept at that temperature for one hr. After being cooled in an ice bath, the amine was extracted with three 150 ml. portions of ether, dried by filtering the extract through anhydrous sodium sulfate and the aniline isolated as the hydrochloride by the introduction of anhydrous hydrogen chloride. There was thus obtained 12.0 g. (91%) of crude aniline hydrochloride.

Dimethylaniline (2)

Excess NaOH solution was added to 11.5 g. of crude aniline hydrochloride prepared as above and the free amine extracted with ether. The solvent was removed, and the residue heated for 16 hr. at 150°C. in a sealed tube with 40 g. of sodium acetate trihydrate, 32 g. methyl iodide, and 20 ml. of water. The reaction mixture was cooled, made alkaline, and steam distilled. Extraction of the steam distillate, followed by drying of the extract, and stripping of the solvent, gave a residue which, upon distillation, yielded 8.5 g. (78.7%) of dimethylaniline, b.p. 190–195°C., $[\text{n}]_D^{25}$ 1.5568. Kjeldahl digestion of the residue from the steam distillation gave 8% recovered ammonia.

DAB (I) (3)

To a stirred solution of 6.5 g. of the crude aniline hydrochloride in 9 ml. of conc. HCl and 50 ml. of water, cooled to 0–5°C., there was added dropwise a solution of 3.9 g. of sodium nitrite in 50 ml. of water. After stirring for 0.5 hr., the excess nitrous acid was destroyed by the addition of urea (starch iodide test paper). To the cold stirred solution there was added 9 g. of dimethylaniline and this was followed one hr. later by the addition of one-half of a solution of 14 g. of sodium acetate trihydrate in 30 ml.

of water. At the end of the second hour the rest of this solution was added and the ice-bath was removed from the reaction vessel. After being stirred for 4 hr. more, the reaction mixture was allowed to stand overnight. The solid precipitate was collected by filtration to give 10.7 g. (95.5%) DAB I, m.p. 113–116°C. A small fraction was recrystallized for analysis from 95% ethanol, m.p. 114–117°C. (corr.). (Lit. 115°, 117°.)

Anal. Calc'd for $C_{14}H_{15}N_2N^{15}$: N, 18.9. Found: N, 18.7, 18.9, 18.8. (N^{15} at.-% excess. Calc'd: 20.2; found: 20.0, 20.1.)

DAB (II)

The reaction was carried out essentially as for DAB (I) using 5.7 g. aniline and 7.3 g. of isotopic dimethylaniline. There was thus obtained 12.4 g. of crude DAB, m.p. 105–108°C., which, on recrystallization from 95% ethanol, gave 11.5 g. (85%) DAB (II), m.p. 112–114°C.

Anal. Calc'd for $C_{14}H_{15}N_2N^{15}$: N, 18.9. Found: N, 18.6, 18.5. (N^{15} at.-% excess. Calc'd: 20.2; found: 20.1, 20.2.)

p-Nitrobenzamide

Using 19.5 g. of *p*-nitrobenzoyl chloride and the ammonia from 11.1 g. of ammonium chloride (N^{15} at.-% excess 61.05), *p*-nitrobenzamide was prepared in a manner analogous to that used for the preparation of benzamide. The ether was removed and the residue recrystallized from alcohol-water, and then water alone to give 16.8 g. (99%), *p*-nitrobenzamide, m.p. 195–198°C. The mother liquors yielded 5.5 g. of recovered isotopic ammonium chloride.

p-Nitroaniline (5)

To an ice-cold solution of 18 g. of bromine and 12 g. of NaOH in 100 ml. of water there was added 16.8 g. of *p*-nitrobenzamide and the mixture was shaken thoroughly for about 10 sec. at one-minute intervals during a period of 10 min. The resulting slurry was added quickly to a stirred and refluxed solution of 20 g. of NaOH in 200 ml. of water. After being refluxed for 30 min., the reaction was allowed to stand overnight. During the reflux period, and for 3 hr. afterward, nitrogen gas was bubbled through the reaction and into a sulfuric acid trap. In this way 0.206 g. (14%) of isotopic ammonia was recovered.

The reaction flask was cooled in ice and the precipitated amine was collected by filtration to give 10.4 g. of *p*-nitroaniline, m.p. 140–147°C. The yield was 75% based on amide used and 89% based on unrecovered ammonia.

p-Nitroacetanilide (6)

Refluxing of 10.4 g. of crude *p*-nitroaniline and 9.0 g. of acetic anhydride in 250 ml. of benzene until crystallization began, followed by cooling in ice, yielded 12.3 g. (91%) of *p*-nitroacetanilide, m.p. 212–214°C. Concentration of the mother liquors gave 0.7 g. more of crude material, m.p. 200–205°C.

N-Acetyl-*p*-Aminodimethylaniline (7)

To 6.5 g. of *p*-nitroacetanilide suspended in 190 ml. of 95% ethanol there was added 8 ml. of 40% formaldehyde, 3.5 ml. of glacial acetic acid and 0.2 g. of Adams' platinum oxide catalyst. During 16 hr. shaking on the Parr apparatus 5 moles of hydrogen were absorbed. After settling, the catalyst was removed by filtration, and the filtrate was combined with that from a similar run in which 5.8 g. of nitro compound was used.

Removal of the solvent, followed by distillation under reduced pressure, gave 9.8 g. (80.6%) of *N*-acetyl-*p*-amino-dimethylaniline, b.p. 180–185°C. at 3 mm., m.p. 122–126°C.

The residue from this distillation was combined with the mother liquors and crude products from the acetylation reaction and the whole was digested by the Kjeldahl method to recover isotopic nitrogen. There was thus obtained 0.403 g. of N, equivalent to 18.9% of the *p*-nitroaniline used in the acetylation reaction.

p-Aminodimethylaniline

N-acetyl-*p*-aminodimethylaniline (9.8 g.) was hydrolyzed by refluxing for 16 hr. in 100 ml. of 3 *N* HCl. The solution was then made alkaline and the amine was isolated by ether extraction.

Removal of the solvent, followed by vacuum distillation, gave 6.9 g. (92.3%) of *p*-aminodimethylaniline, b.p. 93–95°C. at 3 mm.

Analysis of the aqueous layer after ether extraction showed 0.044 g. (2.8%) of nitrogen was present.

DAB (III) (4)

To the solution formed by the mixing of 5.6 g. of nitrosobenzene in 40 ml. of pyridine and 6.3 g. of *p*-aminodimethylaniline in 30 ml. of pyridine there was added 5 ml. of glacial acetic acid. After the initial heat of reaction subsided, the solution was refluxed 10 min. and allowed to stand at room temperature for 2 hr.

The reaction mixture was poured into 500 ml. of ice-water and the precipitate was collected by filtration to give 9.6 g. of crude DAB, m.p. 95–105°, which, on recrystallization from 95% ethanol, yielded 8.3 g. (80%) DAB (III), m.p. 112–115°C.

Anal. Calc'd for $C_{14}H_{16}H_2N^{15}$: N, 18.9. Found: N, 18.6, 18.6. (N^{15} at.-% excess. Calc'd; 20.4; found: 20.4, 20.6.)

Position of N¹⁵ in p-Aminodimethylaniline

Isotopic *p*-aminodimethylaniline (0.45 g.) was diluted with 4.54 g. of normal material. Analysis of the mixture showed N^{15} at.-% excess 2.7, 2.7. This 4.99 g. of amine was diazotized with 2.7 g. of sodium nitrite and 5 ml. of conc. H_2SO_4 in 100 ml. of water. Two ml. aliquots of the diazonium solution were treated with excess NaOH solution and the evolved nitrogen was collected for mass spectrographic analysis. (N^{15} at.-% excess. Calc'd: 2.7; found: 2.8, 2.9.)

Position of N¹⁵ in DAB (I)

Isotopic DAB (I) (0.5 g.) and ordinary DAB (4.5 g.) were mixed by recrystallization from alcohol. The 4.57 g. of recovered DAB (N¹⁵at.-% excess 2.0, 2.1) in 100 ml. of alcohol was reduced on the Parr apparatus in the presence of 0.1 g. platinum oxide catalyst. Two moles of hydrogen were absorbed and the reaction became colorless. The catalyst and solvent were removed in the usual manner and the residue distilled to give 1.3 g. of impure aniline, b.p. 185–190°C. (N¹⁵at.-% excess. Calc'd: 6.0; found: 5.2, 5.2) and 2.6 g. of impure *p*-aminodimethylaniline, b.p. 85–95°C. at 3 mm. (N¹⁵at.-% excess. Calc'd: 0.0; found: 0.85, 0.87.)

SUMMARY

p-Dimethylaminoazobenzene containing N¹⁵ in each of the three possible positions has been synthesized.

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The Effect of Age on Calcium Binding in Mouse Liver ¹

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INTRODUCTION

An increase with age in the calcium content of various plants and animals appears to be well established (4). In an attempt to particularize this calcium of aging, Lansing (1,2,3) has described an increase with age of calcium in the periphery of cells of widely different organisms, and has shown that experimental removal of calcium from the cells of a rotifer resulted in a significant extension of the total life span.

Why does the calcium content of cells increase with age? An answer to this question depends to a large extent on elucidation of the calcium binding mechanisms in cells and their possible changes with age.

The preceding two problems are not attacked with equal readiness. Little is known of the calcium binding materials in cells. Certainly, proteins in general may be expected to bind calcium, and it is possible that one or more proteins characteristically change with age to increase their calcium binding capacities.

The present study deals with changes of calcium binding with age. The method we have employed depends essentially upon quantitatively tracing the distribution of radiocalcium in the livers of young and old mice, using ultrafiltration to separate free and bound calcium.

EXPERIMENTAL

Swiss mice were used in all of these experiments; the growing animals were 2-3 months old (approximately 22 g.) and the old full grown animals were 12-13 months old (approximately 29.5 g.). A total of 1 cc. CaCl_2 solution (1 mg./cc. as Ca and about 50,000 counts/min. as Ca^{45}) was injected intraperitoneally in both age groups. Five animals of each age group were sacrificed by a blow on the head and bled at 6, 12, 18, 24, 30, 36, 48, and 72 hr. after injection of the isotope. The livers were removed and

¹ Aided by a grant from the U. S. Public Health Service.

packed in solid CO_2 until all of the livers had been accumulated for simultaneous ultrafiltration. The latter precaution was taken to insure relatively uniform handling of the material during the ultrafiltration process.

In preparation for ultrafiltration the pooled livers of each time period were allowed to thaw in a cold room maintained at $0.5\text{--}2.0^\circ\text{C}$., ground with mortar and pestle and suspended in normal saline (10 cc./g. of tissue). The tissue suspensions were transferred to collodion sacs and filtered at a negative pressure of 150 mm. Hg and at a temperature of $0.5\text{--}2.0^\circ\text{C}$. over a period of 72 hr. Details of the procedure have been described in a previous publication (6).

Aliquots of both the ultrafilterable and non-ultrafilterable portions were removed for calcium analyses which were performed by methods described in the aforementioned report. Radioactivities were measured using a thin mica window G. M. tube and Tracerlab Autoscaler.

RESULTS AND DISCUSSION

The combination of quantitative calcium analyses and radioactivity measurements with ultrafiltrations for filterable and non-filterable calcium has made it possible for us to differentiate between calcium uptake and calcium turnover in what may be termed, for convenience, the "free" and "bound" tissue calcium. The methods employed are such that it is not possible to conclusively differentiate between intracellular and intercellular calcium. However, since extracellular ions are in ready equilibrium with the tissue fluids and blood it seems reasonable to expect that measurements of Ca^{45} levels in blood should reflect the Ca^{45} in the intercellular spaces.

We have recently shown (5) that, under conditions comparable to those of our present experiments, blood Ca^{45} displays a constant specific activity at approximately 24 hr. after injection of the isotope. Accordingly, we might expect that intercellular Ca^{45} would reach a constant level of specific activity at 24 hr., and any fluctuations in Ca^{45} level thereafter would reflect changes in intracellular Ca^{45} .

Fig. 1. is a graphic representation of the quantitative variations of calcium in the filterable and non-filterable fractions of young and old mouse liver after injection of radiocalcium. It is readily apparent that the data for young and old liver are widely divergent. Total calcium for young liver increases but slightly at 12 hr. after injection of Ca^{45} , recedes at 18 hr., and is relatively constant thereafter. The levels of filterable calcium increase somewhat at 12, 36, and 48 hr. after injection but otherwise remain constant. The data indicate that the total amount of calcium in young mouse liver after injection of relatively

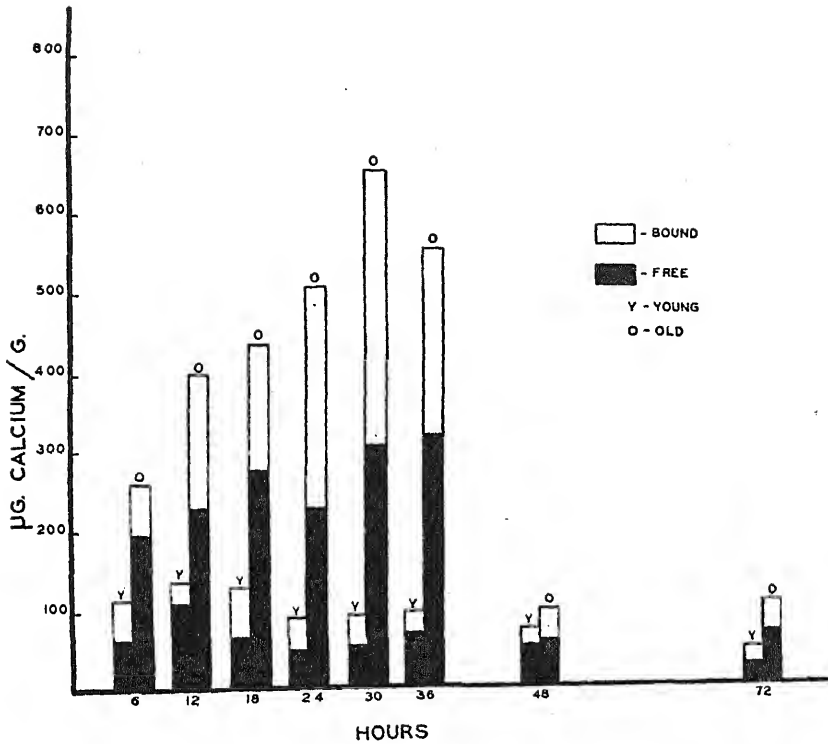


FIG. 1. Histogram showing the relation between free and bound calcium of young and old mouse liver as a function of time after injection of radiocalcium.

large amounts of radiocalcium remains essentially unchanged and, accordingly, there is little uptake of Ca^{45} .

The data for old liver indicate a strikingly different sequence of events. As early as 6 hr. after injection of the isotope the total calcium level begins to increase sharply to a maximum of 660 γ Ca/g. at 30 hr. It decreases somewhat at 36 hr. and reaches basal levels at 48 and 72 hr. after injection.

It will be noted that a disproportionately large portion of this calcium increase is consistently in the bound calcium fractions. These results show that the calcium uptake by old liver subsequent to a large dose of intraperitoneally injected calcium is at a high level and that a significantly greater portion of this uptake is in the bound fraction.

The specific activities of the free and bound calcium in young and old mouse liver at the various time intervals after injection of Ca^{45} are shown in Fig. 2. From previous data we have determined that the specific activities prior to the 24 hr. period reflect the physiologic imbalances resulting from the large dose of calcium employed. As mentioned before, blood calcium reaches normal levels at 24 hr. after injection. Significant data are considered as those obtained after the 24 hr. period. Here again, the data for young and old liver differ

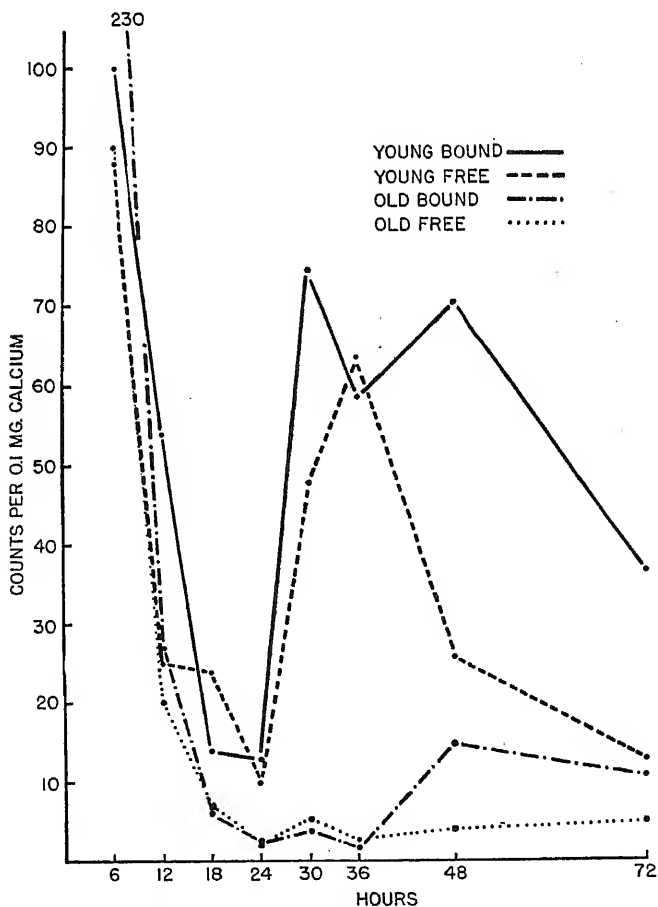


FIG. 2. Graph showing the changes in specific activity of free and bound calcium (Ca^{45}) of young and old liver after injection of radiocalcium.

basically. The specific activities of free and bound calcium in young liver increase abruptly after 24 hr. and remain at a maximum through 36 hr. Both free and bound calcium data are parallel until this time. The specific activity of the free calcium falls off sharply at 48 hr., at which time the bound calcium value is still maximal. At 72 hr. the specific activity of the bound calcium is more than 100% greater than that of the free.

The specific activities of free and bound calcium of old liver remain at a very low level through the 36 hr. period. Thereafter, while the free calcium activity continues unchanged through 72 hr., the bound calcium specific activity increases significantly through the 48 and 72 hr. periods.

These data indicate that Ca^{45} turnover in both the free and bound calcium of young mouse liver is at a high level. In contrast, turnover of calcium in the bound fraction of old liver is relatively low, and that of the free fraction of old liver is minimal.

It appears then that young and old mouse liver differ significantly in the following respects:

- (1) Young mouse liver is unable to take up or store significant amounts of calcium.
- (2) Calcium exchanges readily in both the free and bound fractions of young liver.
- (3) Old mouse liver is capable of storing large amounts of calcium, particularly in the bound fraction.
- (4) Calcium that is bound in old liver is not as labile as that of young liver; its turnover is much lower.
- (5) Free calcium turnover in old liver is minimal.

One of us (A.I.L.) has initiated a program of study on the nature of the calcium binding. Results of preliminary experiments are of interest in that they suggest that calcium may be largely bound to a lipide as well as a ribonucleoprotein complex. Thus, alcohol-ether extraction of frozen and dried liver removes a significant amount of calcium, and the amount of such extractable calcium is greatly increased in aging. The amount of filterable Ca^{45} labeled calcium obtained by ultrafiltration from young liver is increased by pepsin, trypsin, and possibly ribonuclease, but not by desoxyribonuclease. Microincinerated preparations of ribonuclease-digested mouse liver show a loss of the usually encountered ring of calcium in the periphery of the hepatic cells, and sections stained with pyronine Y show a complete absence of the stain in the cell periphery as well as a pronounced reduction in the amount of stain taken up by the remainder of the cytoplasm.

SUMMARY

The distribution of Ca^{45} -labeled calcium in livers of young and old mice was investigated, ultrafiltration being used to separate free and bound calcium.

The data indicate that in young tissue the calcium uptake is low and turnover is rapid. In old tissue the calcium uptake is high and turnover is low.

The suggestion is made that intracellular calcium may be largely associated with a lipide and a ribonucleoprotein complex.

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Phytohormones: Structure and Physiological Activity. II ¹

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In an earlier paper (3), the molecular configuration, common to all substances known to cause growth by cell elongation in plants, was defined as follows: an unsaturated ring system with a side chain carrying a carboxyl group (or a group easily hydrolyzed to a carboxyl group) at least 1 carbon atom removed from the ring, with a special space relationship between carboxyl group and ring. In the intervening years a number of papers have appeared substantiating, modifying or refuting these minimal structural requirements.

Leech (5) studied a number of aliphatic monocarboxylic acids, none of which had growth activity. Gandini (2) found that santonic acid, cyclohexanone-2-acetic acid and 4-methylcyclohexanone-2-acetic acid were active in the pea test. This made him conclude that keto-enol tautomerisation occurred in these substances, producing a double bond in the ring system adjacent to the side chain carrying the carboxyl group, and thus these substances agreed with the structural prerequisites suggested by Koepfli *et al.*

Veldstra (9,10) could confirm the conclusions of Koepfli *et al.* but restated the requirements for a plant growth substance as follows:

(1) The substance should have a basal ring system with high surface activity, and (2) it should have a carboxyl group in a very definite spatial position with respect to this ring system.

Zimmerman and Hitchcock (13) have described the activity of many substances on a variety of plants when applied in a variety of ways, and made it their "purpose to point out the inconsistencies and the difficulties encountered in attempting to arrive at a rational basis for

¹ The author gratefully acknowledges the help of Mrs. G. M. Wilson in carrying out part of the pea tests for this investigation.

selecting physiologically active substances by means of a study of their structural formulae." The discrepancies of their active substances with the minimal structural requirements as set forth by Koepfli *et al.* lie in the differences in tests used. They found it necessary to distinguish between activity as measured by bending responses of stems and by modification of organs. They failed, however, to distinguish between growth activity and preparatory activity as advocated by Went (12) and as practiced by Koepfli *et al.*

In the present paper are reported the growth activities of a few new substances, synthesized by Dr. J. B. Koepfli, and a general survey of the activity of substances in the preparatory reaction.

EXPERIMENTAL

Methods

The pea test was used throughout this investigation and a distinction was made between preparatory activity and growth activity proper (11). To this end, the growing regions of pea stems, grown in a darkroom at 25°C., were slit and laid to soak in water for 2 or 4 hr. Then they were transferred to the pretreatment solutions, where they were left for 2 hr., and finally they were laid in weak solutions of indoleacetic acid. The curvatures were measured 20 hr. later from the inflection point. The activity of substances in the preparatory reaction was judged by the increase in the response to indoleacetic acid, especially the 0.2 mg./l. concentration, when indoleacetic acid has very little preparatory activity.

To test for primary growth activity, pea stems are slit as described before, and after 2-4 hr. of water treatment are placed in a weak solution of the substance. If curvatures appear within 24 hr., the substance has primary growth activity. To discover whether there exist substances, possessing primary growth activity but not preparatory activity, slit peas are pretreated for 2 hr. with a concentration of 50 or 100 mg./l. of one of the more active hemiauxins (substances with preparatory activity only, such as γ -phenylbutyric acid, cyclohexanecarboxylic acid or 2-bromo-3-nitrobenzoic acid). Then they are transferred to the solutions to be tested. If curvatures appear in the pretreated peas only, the active substance lacks preparatory activity.

Only substances at least doubling the activity of a 0.2 mg./l. solution of indoleacetic acid are considered in this paper. In the course of this investigation well over 10,000 pea stem halves were measured. Every substance was tested at least twice on different days.

Primary Growth Activity

To narrow down the structural requirements for growth activity, some critical compounds were prepared to study the space relationships between carboxyl group and double bond. Whereas 1-cyclohexene-1-carboxylic acid is active, 2-cyclohexene-1-carboxylic acid is completely inactive. This is not due to the increased distance between

carboxyl group and double bond, because phenyl propionic and indolepropionic acids are active. Similarly 2-cyclohexene-1-carboxylic acid is inactive, and so is 2-cyclohexene-1-propionic acid. Therefore, this series of compounds shows that growth activity proper is exhibited only by substances in which a side-chain of at least two carbon atoms is placed adjacent to the double bond in the ring.

Although many substances were tested, not a single one was found which does possess growth activity proper but which does not have preparatory activity. This was tested by preparing the peas in the standard way with cyclohexane-acetic acid of γ -phenylbutyric acid, and then placing them in a solution of the substance to be tested.

Preparatory Activity

As published earlier (11,12), substances active in the pea test must take part in 2 successive reactions: first, the preparatory reaction and then the growth reaction proper. These two reactions are fundamentally different, as can be seen by the separation in time, the preparatory reaction preceding the growth reaction, the difference in required concentration for optimal action, the difference in pH sensitivity, and the difference in structural requirements. Cyclohexaneacetic acid, for instance,^f has preparatory activity, but not growth activity. The following list enumerates all substances found up to the present to have preparatory activity only (hemiauxins). The substances marked with an asterisk were tested by Leech (5), and those marked with a dagger by Datta (1).

γ -Phenylbutyric acid, cyclohexaneacetic acid, 2-cyclohexene-1-acetic acid, 2-cyclohexene-1-propionic acid, thiazoleacetic acid, *imidazoleacetic acid, *benzimidazoleacetic acid, †2-bromo-3-nitrobenzoic acid, †2-bromo-3,5-dichlorobenzoic acid, 2-chloro-3-nitrobenzoic acid, *vinylacetic acid.

The most interesting result of this work is that all substances exhibiting preparatory activity only (hemiauxins) are structurally closely related to growth-promoting substances (auxins). Substances having preparatory activity fall in the following general groups:

1. Auxins. All substances having growth activity proved to have preparatory activity as well. This is easy to show in the case of relatively inactive substances, but is difficult to measure in substances which are very active in the pea test (such as naphthaleneacetic acid).

2. Substances which, because of their structure, would be expected to have growth activity, yet fail to show any growth activity proper. Examples of such substances are γ -phenylbutyric acid and thiazoleacetic acid.

3. Substances similar to auxins in all respects except the ring double bond. Examples are cyclohexaneacetic acid, 2-cyclohexene-1-acetic acid, and 2-cyclohexene-1-propionic acid.

4. Substances similar to auxins in all respects except the length of the side-chain. Among the latter substances there is no carboxylic acid. But several substituted benzoic acids are very active as hemiauxins, such as 2-bromo-3,5-dichlorobenzoic acid.

5. Substances having a double bond in the 3-position from the carboxyl group, but not possessing a ring system, such as vinylacetic acid (5).

6. It is possible that substances like ethylene have to be grouped under the hemiauxins. The action of ethylene is in many respects comparable to that of γ -phenylbutyric acid (6), but in the standard pea test it is inactive, so that for the present it can be omitted from our considerations.

That for the rest there is a very definite specificity for the preparatory reaction, will be seen from the following list of definitely inactive substances. Most of these have been tested over a range of 10–500 mg./l., some as low as 0.5 mg./l.

Aliphatic Acids: Acetic, butyric, valeric, citric, glycollic, suberic, malonic, fumaric, pimelic, succinic, adipic, glutaric, furoic, *acrylic, *crotonic, *sorbic, and * α -methyl- β -ethylacrylic acids.

Amino Acids: DL-valine, arginine, alanine, D-glutamic acid.

Aromatic Acids: benzoic, phenylglyoxylic, α -phenyl- β -chloropropionic, pyrrolecarboxylic, methylpyrrolecarboxylic, anthranilic, anthraquinonesulfonic, naphthol-4-sulfonic, nicotinic, quinic, 2-pyrimidineacetic, indolecarboxylic, *trans*-cinnamic, cyclohexanecarboxylic, 2-cyclohexene-1-carboxylic, *l*- and *d*-mandelic (?), α -methyl- α -aminophenylacetic, α -chlorophenylacetic, α -phenylisobutyric, tropic (?), *m*- and *p*-nitrocinnamic (?) acids.

Nonacids: ethyl benzoylformate, salicylideneacetamide, adenine, nicotinamide.

The substances marked with a question mark seemed to have slight activity in some tests; none, however, on other days. Both tropic and mandelic acids, of which the inactivity as growth substances was considered remarkable in the first paper, have questionable activities, so that the presence of one hydroxyl group close to the carboxyl group at least greatly reduces activity.

DISCUSSION

The objections raised by Zimmerman and Hitchcock (13) against the minimal structural requirements for a growth-promoting substance as formulated by Koepfli *et al.* (3) were especially along two lines: the requirements could be met by substances which were inactive, and some substances, specifically substituted benzoic acids, were active in their tests without possessing the 2 carbon atom side-chain.

The first objection is not real, since it had been pointed out that substitution, either in the nucleus or the side-chain, could inactivate substances otherwise active (3,4). Besides, it was only stated that substances must possess the minimal structural requirements to be active, and not that all substances having the specified structure were active in growth.

It now has been shown that the other objection is not real either, because, when the proper precautions are taken, substituted benzoic acids are inactive in producing growth (2-bromo-3-nitrobenzoic acid is also completely inactive in the *Avena* test). Since many of them are

active in the preparatory reaction, they would activate the naturally occurring auxin in the test objects of Zimmerman and Hitchcock, and in that way would seem to be active in promoting growth.

It seems to be necessary to point out again, that structural specificity of physiologically active substances can only be expected to be found when the test for specificity is specific. And only if a specific reaction or process is isolated does an analysis of specificity have a theoretical meaning.

From the enumeration of the substances active as hemiauxins, it is clear that no minimal structural requirements can be given, other than that all are organic substances with a carboxyl group. Even this requirement is probably unessential, since Veldstra (10) has published activity of a sulfonic acid. They either have a double bond or not, they either have a ring or not, they either are carboxylic acids or have longer side-chains with a carboxyl group. Yet a clear-cut statement can be made. All substances having preparatory activity in the pea test are acids which do not lack more than one of the minimal structural requirements for auxins. Substances differing in more than one respect from auxins, such as cyclohexanecarboxylic acid and butyric acid, never have been found active as hemiauxins. This also explains why no substances were found to possess growth activity but not preparatory activity: any substance active as a growth substance possesses the structural requirements for preparatory activity.

Until more critical compounds have been tested, the activating effect of substituents in benzoic acid cannot be discussed. Chlorine substitution in the side chain of phenylacetic acid results in an inactive compound, whereas chlorine substitution in the benzene nucleus of phenoxyacetic acid causes activation (see 1).

Thimann and Schneider (7) have stated that γ -phenylbutyric acid had a slight growth activity in the pea test. This must have been due to small amounts of natural auxin being activated, because in the thousands of peas treated in Pasadena the lack of growth activity proper of γ -phenylbutyric acid has been unequivocal.

Many substances reported active in 3 tests by Thompson *et al.* (8), and possessing growth inhibitory effects, have the basic structure of auxins. This would lead us to believe that growth inhibition (or popularly called weed-killing action) is connected with an auxin-like structure. This is not correct, however, since many substituted benzoic acids, possessing no growth activity whatever, are listed among the

active substances by Thompson *et al.* Since it was found that these substituted benzoic acids possess preparatory activity, we can say that both auxins and hemiauxins are effective in growth inhibition. It had been concluded earlier [(11), point 5 on p. 591] that for correlative bud inhibition preparatory activity, but not growth activity, is required. Therefore, we might suggest that in weed killers we should measure preparatory activity and try to correlate this with effectiveness as weed killers. Would the other substances reported here as hemiauxins also be effective as weed killers (for instance γ -phenylbutyric acid or cyclohexanecarboxylic acid) or are there special requirements, such as ease of transport through the plant, which are not fulfilled by hemiauxins in general?

SUMMARY

The minimal structural requirements for a substance to exhibit growth activity in the pea test can be made somewhat more precise, by stating that the substance must have an unsaturated ring system, with a side chain, *adjacent* to the ring double bond, of at least 2 carbon atoms ending in a carboxyl group. For preparatory activity the same structural requirements hold, except that any single one of the specifically listed requirements can be absent, provided all others are satisfied. Thus, either the ring, or the double bond, or one of the two carbon atoms in the side chain can be missing.

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Specificity and Purification of Polygalacturonase

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INTRODUCTION

Pectic enzyme preparations have received increased attention in recent years because they promote filtration and accelerate clarification of fruit juices and wine, and because they catalyze the hydrolysis of pectin to low methoxyl pectins, pectic acid, and galacturonic acid. The latter has been proposed as a practical starting point for the manufacture of ascorbic acid. In spite of the interest in pectic enzymes,³ the non-availability of purified pectic enzymes and lack of information on specificity prevent rigorous interpretations of their role in commercial processes and in the physiology of ripening in the plant kingdom, at least in so far as texture changes are concerned.

Crude pectic enzyme preparations of course contain numerous enzymes besides polygalacturonase and pectinesterase (pectase). With regard to glycosidases, Pigman (4) showed that Pectinol 100 D⁴ catalyzes the hydrolysis of sucrose and inulin, and Fish and Dustman (5) showed that Pectinol A⁴ catalyzes the hydrolysis of starch, sucrose, maltose and inulin, but not xylan. These authors did not imply, of course, that the activities were due to either the polygalacturonase (PG) or the pectinesterase (PE) of the pectic enzyme preparations. In fact, it is reasonable to suppose that PG is highly specific as are polysaccharidases such as the amylases. The data of Jansen and MacDonnell (6) are pertinent in this connection. They showed that PG, which

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³ For recent reviews of pectin and pectic enzymes, including practical applications, see Refs. 1 and 2; also see earlier review, Ref. 3.

⁴ Pectinol 100 D and Pectinol A are two different dilutions of the fungal enzyme preparation, Pectinol 46 AP, prepared by Rohm and Haas Co. Pectinol 46 AP when diluted to standard strength with diatomaceous earth is Pectinol 100 D and when diluted with soluble materials is Pectinol A.

rapidly hydrolyzes pectic acid, hydrolyzes esterified pectic substances (*i.e.*, pectin and pectinic acids) to only a limited extent. Thus, free carboxyl groups in the substrate were shown to be essential for PG action. This finding suggests that nonuronic acid compounds would be refractory to PG but leaves the real, though unlikely, possibility that uronic acid compounds other than pectic acid might be hydrolyzed by PG. The possibility, however, has not been realized experimentally.

Our results show that PG is unable to hydrolyze nonpectic substances. Substances tested included 11 that are related to pectin by virtue of their content of uronic acid or galactose. Failure of PG to hydrolyze one of these substances, the methyl α -glycoside of galacturonic acid, indicates that PG is not an enzyme that hydrolyzes simple glycosides. It is of special interest that pneumococcus polysaccharide (Type I), gum tragacanth, and gum ghatti, which are reported to contain some galacturonic acid residues, are not hydrolyzed by PG (and therefore do not possess the pectic acid-type glycosidic structure). Obviously the high specificity of PG recommends it as a tool in studies of the structures of complex substances and in studies of the composition of biological materials.

The principal purpose of this paper is to describe the specificity of PG; also included are a description of certain properties of the two crude fungal enzyme preparations^{5,6} used as sources of PG and a description of two methods of purifying PG.

METHODS AND MATERIALS

Polygalacturonase Assay

Glycosidic hydrolysis of pectic substances was determined at 25°C. and pH 5 by a modified Willstätter-Schudel hypiodite method (6). One unit of hydrolytic activity is the amount that will release one millimole of reducing groups (or galacturonic acid)

⁵ Crude pectic enzyme preparations [pectinase (3,7)] that contain both PG and PE are prepared generally from fungi, although other microorganisms and certain higher plants produce them too. For convenience, especially with regard to purification studies, we have used 2 commercially available enzyme preparations, Pectinols 45 AP and 46AP manufactured by Rohm and Haas Co. (*cf.* footnote 4 and refs. 4 and 8). It is thought that Pectinol 45 AP and Pectinol 46 AP are grossly representative of fungal pectinase. However, since differences occur in certain of the properties of these preparations, broad generalizations based on the results reported here must be qualified. The specificity did not differ.

⁶ The mention of this product and other commercial products and firm names does not imply endorsement or recommendation by the Department of Agriculture over other products of a similar nature and other firms not named.

per min. under the conditions described by Jansen and MacDonnell (6). The symbol $[\text{PG.u.}]_{\text{mg.TN}}$ will be used to express the units/mg. total nitrogen, and corresponding symbols will be used to indicate units/ml., mg., etc.

Pectinesterase Assay

The liberation of carboxyl groups of pectin was determined at pH 4.0 and 30°C. in the presence of 0.05 *M* Ca^{++} by titration with 0.02 *N* NaOH as previously described (6). One unit of activity is the amount that releases one milliequivalent of acid/min. under the defined conditions. Symbols similar to those used for PG will be used to express the activity, e.g., $[\text{PE.u.}]_{\text{mg.TN}}$.

Assays for Other Enzyme Activities

The hydrolysis of the various substrates with crude and purified enzyme preparations was determined at pH 5. The increase in reducing power was measured at times ranging from 10 min. to 24 hr., depending on the rate of hydrolysis of the substrate. For long runs, 0.5% toluene was added as preservative. The substrate concentrations used were between 0.25 and 1.0%. Starch, maltose, sucrose, and inulin were assayed in the presence of 0.2 *M* acetate buffer and 0.2 *M* NaCl; all other substrates were assayed without either salt or buffer. All determinations were made at room temperature (23–25°C.) except for amylase, which was run at 37°C.

All substrates used in the specificity studies were unpurified commercial preparations except the following, which were prepared or obtained as indicated: Heparin was purified by precipitation with 9 vols. of glacial acetic acid, centrifugation, washing of the precipitate with ethanol and ether, and final drying in a desiccator over CaCl_2 . The methyl glycoside of polygalacturonic acid methyl ester and methyl α -D-galacturonide methyl ester were prepared according to the methods of Morell, Baur and Link (9), and Morell and Link (10), respectively. The acids corresponding to these esters were obtained by saponification of the esters. Pectic acid was prepared from Pectinum NF VII with orange pectinesterase (6). Ovomucoid was prepared by alcoholic precipitation of the soluble protein remaining after removal of heat-denaturable protein present in the filtrate from egg albumin crystallization. *Pneumococcus* polysaccharide, *Type I*, was given to us by Dr. J. W. Palmer, and the hyaluronic acid was given to us by Dr. N. W. Pirie. For the substrates that were rapidly hydrolyzed, hydrolysis times ranging from 8 to 90 min. and 0.2 to 0.3 unit of PG/50 ml. substrate were used generally. For the other substrates, hydrolysis times ranging from 17 to 24 hr. and 0.1–0.2 unit of PG/50 ml. substrate were used.

Total Solids

A weighed aliquot of the enzyme preparation in a tared wide-mouth weighing bottle was evaporated to dryness within 1.5 hr. in a forced-draft oven at 95°C. Drying was completed in an oven at 82–83°C. until constant weight was obtained (usually less than 9 hr.).

Total and Protein Nitrogen

Total nitrogen was determined by the micro Kjeldahl method with mercuric oxide as catalyst. Protein nitrogen values were not used, because the nondialyzable nitrogen is appreciably soluble in 2.5% trichloroacetic acid, even for highly purified PG.

Enzymes

Pectinase preparations manufactured by Rohm and Haas Co. and designated Pectinols 45 AP and 46 AP were used in this study (*cf.* footnotes 4,5, and 6).

RESULTS

Properties of Crude Pectinases that Relate to the Purification of PG

Under this heading we will describe (a) the influence of dialysis on the specific activities of the enzyme preparations, (b) the relative pH stability of PG and PE, and (c) some of our observations on the influence of the resin IR-100 on PG and PE.

Previous reports (6,11,12) on the purification of PG concern almost exclusively the separation of PG and PE. Jansen and MacDonnell (6), following the basic observations of Rothschild (11) that PE is less stable in acid solution than PG, obtained PG preparations with so little PE in them that it was easy to demonstrate quantitative dependence of PG action on degree of deesterification of pectin. McCulloch and Kertész (12) used an ion exchange resin for removal (or inactivation) of the PE component of pectinase.

The specific PG and PE activities and the nitrogen contents before and after dialysis are reported in Table I for the single lot of 45 AP and for one of the lots of 46 AP that were used in this study. Dialysis was carried out in collodion membranes, since the Pectinols contain an enzyme, possibly a cellulase, that destroys Cellophane membranes. About two-thirds of the solids of 45 AP, and about half of the solids of 46 AP passed through the collodion membrane. The two enzyme preparations as

TABLE I
*Specific PG and PE Activities of Pectinols 45 AP and 46 AP
Before and After Dialysis*

Enzyme sample	Nitrogen	PG. u. per mg. TN	PE. u. per mg. TN	Ratio PG/PE
	<i>per cent</i>			
45 AP	6.1	0.21	0.0021	100
45 AP, dialyzed	7.9	0.46	0.0052	89
46 AP	5.5	0.058	0.0032	18
46 AP, dialyzed	5.9	0.112	0.0059	19

received contained similar amounts of PE, but 45 AP contained about 4 times as much PG as did 46 AP on both a solids and a nitrogen basis (compare ratios of PG to PE in Table I).

Acid conditions cause much more rapid inactivation of the PE component of 45 AP than of 46 AP (Table II, runs No. 5 and 8 compared

TABLE II
*Effect of pH and Temperature on Stability of PG and PE
in 45 AP and 46 AP*

Enzyme sample	Run no.	pH	Temp.	Time	Residual activity	
					PG	PE
Untreated 46 AP ^a	1	0.5	°C	min.	per cent	per cent
	2	0.6	5	10-60	80-70	40-30
	3	0.6	5	2.5	100	77
	4	0.6	15	20	56	25
	5	0.6	RT ^b	20	30-50	15-20
	6	1.8-1.9	RT ^b	30	55-60	50-60
	7	2.5	35	10	50	80
Collodion dialyzed 46 AP	8	2.5	45	10	3	40
	9	3.0	RT ^b	30	71	77
Pb-treated 46 AP	10	2.5	RT ^b	30	85	77
	11	0.6	RT ^b	2	27	<0.01
	12	0.6	RT ^b	4	18	<0.01
Untreated 45 AP ^a	13	2.0	RT ^b	2	50	27
	14	1.8	RT ^b	30	47	<0.4
	15	2.1	RT ^b	30	77	<0.4
	16	2.5	RT ^b	30	71	21
	17	3.0	RT ^b	30	100	69
	18	3.0	RT ^b	3 hr.	—	87 ^c
Alginic acid eluate of 45 AP	19	3.0	RT ^b	22 hr.	—	14
	20	3.15	RT ^b	2 hr.	100	37
	21	3.15	RT ^b	5 hr.	94	15
	22	3.15	RT ^b	22 hr.	76	<0.8
	23	3.20	RT ^b	18 hr.	95	2
	24	3.62	RT ^b	3 hr.	100	93
	25	3.62	RT ^b	22 hr.	85	46

^a The specific activities for the untreated enzyme preparations are given in Table I.

^b Room temperature, $23 \pm 1^\circ\text{C}$., was used in these cases.

^c This figure is probably in error.

with No. 13 and 15). However, acid conditions cause about the same rate of inactivation of the PG components of the two preparations. It is evident, therefore, that the PG can be freed of PE more readily for 45 AP than for 46 AP (compare runs No. 3, 4, and 5 with 13 and 14.) However, 46 AP was used in our first studies because it was used in fruit-juice clarification studies and in the preparation of galacturonic acid and because of its availability. Pectinol 45 AP, an experimental preparation, was made available later through the courtesy of Rohm and Haas Co.

A limited study of the influence of temperature on stability of PG and PE of 46 AP showed that PG was inactivated more rapidly at 35–45°C. than PE (runs No. 6 and 7, Table II). The possibility of obtaining PE essentially free of PG by taking advantage of this property is indicated.

TABLE III

Effect of Impurities from Pectinol 46 AP on the Stability of Pectinol 45 AP

Enzyme ^a	Total units of PE × 100				
	pH 1.8	pH 2.0		pH 3.0	
	30 min.	2 min.	10 min.	5 hr.	22 hr.
1. 46 AP	2.7	3.0	2.9	4.3	3.3
2. 45 AP	<0.01	0.5	<0.02	1.8	0.3
3. 46AP + 45 AP		3.1	2.8	6.3	3.5
3a. 46 AP + 45 AP (calc.)		(3.5)	(2.9)	(6.1)	(3.6)
4. Heated 46 AP + 45 AP	<0.01				
4a. Heated 46 AP + 45 AP (calc.)	(<0.01)				

^a Mixtures were made as follows; (1) 1 vol. 46 AP + 1 vol. of H₂O; (2) 1 vol. of 45 AP + 1 vol. of H₂O; (3) 1 vol. of 46 AP + 1 vol. of 45 AP; (4) 1 vol. of heated 46 AP + 1 vol. of 45 AP.

The stability of PG and PE at acid pH values differed with different lots of 46 AP (only one lot of 45 AP was used), but in no case was the PE of 46 AP nearly as sensitive to acid pH as was that of 45 AP. Perhaps the varying behavior of lots of Pectinol explain some of the differences in our results and those of McColloch and Kertész (12).

Purification of Pectinols resulted in increased sensitivity of PE to acid pH. The difference in stability of PE in the two Pectinols is not, however, due to different degrees of purity (Table III). When 45 and

46 AP were mixed, the observed retention of PE was the same as that calculated from separate exposure of the enzyme preparations to the selected pH values.

TABLE IV
Comparison of Effect of pH on PG and PE in Presence and Absence of IR-100

Enzyme	pH (final)	Residual PG after treatment with		Residual PE after treatment with		PG. u./mg. TN	
		Acid only	IR-100	Acid only	IR-100	Orig.	After IR-100
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		
45 AP ^a	1.6 to 1.8	—	41 to 55	—	<0.4	0.24	0.53 to 0.62
45 AP ^b	1.8	47	50	<0.4	<0.3	.24	.37
45 AP ^c	2.1	77	75	<0.4	<0.4	.24	.36
46 AP ^a	1.8	54	40	50	52	.06	.12

^a These samples were treated successively with 25, 15, and 10 g. of dry IR-100/100 ml. original volume of 2% extract of enzyme. These manipulations required about 30 min. and the acid treatments were for about 30 min. The pH values after the first, second, and last treatments were 2.0, 1.9, and 1.8, respectively, for both 45 AP and 46 AP, except in one case, where a final pH of 1.6 was obtained.

^b This sample was treated as described in above footnote, except that 8, 5, and 3 g. of IR-100 were used.

^c This sample was treated as in footnote a, except that 2.5, 1.5, and 1.0 g. of IR-100 were used.

The results reported in Table IV represent part of the data obtained in attempts to remove PE from Pectinol by the use of the ion exchange resin IR-100 as recommended by McColloch and Kertész (12). So far as activities of PE and PG were concerned, the same destruction was obtained in the absence of IR-100 as in its presence when the pH values of the solutions were the same. It should be noted that McColloch and Kertész failed to remove PE from 46 AP unless potassium acid phthalate was added to the solution. Our two lots of 46 AP did not lose their PE quantitatively when treated with IR-100 even in the presence of potassium acid phthalate (unreported data). Although this result may have been due to differences in the lot of enzyme used or to slight differences in technique, the procedure was not satisfactory for our purpose, even though the specific activity per mg. of total nitrogen was increased 50 to 100% by the IR-100 treatment (last two columns of Table IV). In this respect 45 AP and 46 AP were similar. Treatment of

46 AP with IR-100 prior to the steps listed in the procedure described below did not result in PG with a higher specific activity than when the adopted procedure alone was used.

Purification of PG

The chief object of the purification work was to obtain preparations that were pure with respect to enzyme activity. However, considerable purification on a solids and nitrogen basis was also accomplished. Thus, 45 AP was purified about 7-fold on a nitrogen basis and about 10-fold on a solids basis, while 46 AP was purified about 30-fold on a nitrogen basis and about 20-fold on a solids basis. Roughly half of the purification was due to the removal of dialyzable solids. The most highly purified PG obtained from 45 AP and from 46 AP had the same specific activity, 1.7 units/mg. of nitrogen. Since different methods of purification were used, this result would suggest that the preparations were nearly pure were it not for the fact that the PG from 45 AP contained 9.6% nitrogen and various preparations of PG from 46 AP contained 4–5% nitrogen. On the other hand, one preparation of PG (from 46 AP) that had 1.3 PG. u./mg. TN showed two main components by electrophoresis. The component (about half the total) that did not migrate at pH 5.5 appeared to contain most of the PG activity.

The first method of purification, which was developed for use with Pectinol 46 AP, gave undesirably low PG yields (3–5%). The other procedure, which is applicable to 45 AP but not to 46 AP, gave PG yields of 20–40%. The principal step in the latter procedure involves the use of alginic acid as an adsorbent. The PG of the two enzyme preparations differs in that the PG of 45 AP is efficiently adsorbed on alginic acid, whereas the PG of 46 AP is adsorbed very inefficiently.

A. Procedure for Purification of PG from Pectinol 46 AP

This procedure involves acid treatment followed by ammonium sulfate and lead acetate fractionation. The filtrate from a 20% extract of Pectinol 46 AP ([PG. u.]_{mg. TN} = 0.04–0.06) is brought to pH 0.6 with 4 *N* HCl, allowed to stand for 20 min. at 25°C., and readjusted to pH 5 with 5 *N* NaOH (PG yield, 50%; PE yield, 25%). The enzyme is precipitated at 0.9 saturation with ammonium sulfate, filtered with the aid of diatomaceous earth (Celite), and dissolved in about one-tenth of the original volume of water. The acid treatment is repeated (yield on original basis: 25% of PG and 5% of PE). Impurities with small amounts of PG but with considerable PE are precipitated at 0.7 saturation with ammonium sulfate. The

residual PG is precipitated by 0.9 saturation with ammonium sulfate (yields: 10% of PG, 0.2% of PE). The precipitate is dissolved and dialyzed in the cold for not more than 24 hr. if Cellophane membranes are used. If longer times are used, the traces of "cellulase" present may cause the Cellophane membranes to weaken and break. The dialyzed solution is then treated with a saturated solution of basic lead acetate. Small aliquots of lead acetate are added and the suspension centrifuged after each addition until it is evident that most of the color has been precipitated. The filtrate is freed of excess lead by addition of dipotassium phosphate (3-5% PG yield; [PG. u.]_{mg.} TN = 1.3-1.7 generally). Addition of too much lead acetate causes excessive precipitation of PG with the impurities. In such cases, the precipitate may be suspended in water and treated with phosphate; the filtrate is then dialyzed and lead-treated as was the original.

B. Procedure for Purification of PG from Pectinol 45 AP

This procedure involves the adsorption of PG on alginic acid and elution with NaCl solution at 5°C. followed by acid inactivation of PE, which is also adsorbed and eluted. A cold (5°C.) 5% extract of Pectinol 45 AP is adjusted to pH 3 with 4 *N* HCl and stirred in an ice bath with freshly washed alginic acid (0.1 g./unit of PG) for about 5 min. The alginic acid, on which the PG is adsorbed, is collected by centrifugation in the cold. Impurities (and a trace of PG) are eluted by washing with half the original volume of cold 0.1 *M* NaCl. The PG is eluted with a fifth of the original volume of cold 1 *M* NaCl. The eluate, which has a pH of 2.2-2.3, is adjusted to approximately pH 5 immediately to prevent inactivation of PG. The elution is repeated 2 or 3 times with convenient volumes of cold 1 *M* NaCl. To inactivate PE, the combined eluates are adjusted to pH 3.2, allowed to stand at room temperature (23 ± 1°C.) under toluene for 18 hr., and readjusted to pH 5. The solution is dialyzed and dried by lyophilization, unless the adsorption cycle is to be repeated.

Table V gives the activity yields of PG, PE, "cellulase,"⁷ and amylase and the specific activities of PG and PE at various stages of the purification procedure. About 50% of the PG and PE were recovered in the combined 1 *M* NaCl eluates, whereas very little of the "cellulase" and amylase, which were probably inactivated, was recovered. The 3-fold purification achieved by adsorption and elution was increased an additional 2-fold by dialysis. The dialyzate on an activity basis was about half as colored as the original extract. Readsorption, elution, and dialysis of the final dialyzate described in Table V gave a product with a specific activity of 1.67 PG. u./mg. TN, but the yield corresponded to only 15% of the original activity; that is, a rather high loss (64%) occurred in this step. Further studies of the purification steps were not attempted, since the purified enzyme hydrolyzed pectin at least 5000 times as fast as it did carboxymethylcellulose, starch, and the methyl ester bonds of pectin, as will be noted later.

⁷ "Cellulase" is used to designate the enzyme that hydrolyzes carboxymethylcellulose. Whereas this may not be strictly justified, the enzyme preparation is able to hydrolyze Cellophane and filter paper—the latter very slowly (unreported data).

TABLE V
Purification of PG of Pectinol 45 AP with Alginic Acid Adsorptoin at 5°C.

Fraction	PG		PE		"Cellu- lase"	Amylase
	Activity yield ^a	Units/mg. TN	Activity yield ^a	Units/mg. TN	Activity yield ^a	Activity yield ^a
Original	per cent 100	0.24	per cent 100	× 100 0.25	per cent 100 ^b	per cent 100 ^b
Supernatant from alginic acid adsorption step	20	0.07	21	0.08	6	16
0.1 N NaCl eluate	<0.2	—	<0.2		0.5	0.2
1 M NaCl eluate I	26	0.83	29	0.94	.9	0.1
1 M NaCl eluate II	18	0.76	18	0.81	.8	<0.01
1 M NaCl eluate III	8	0.71	2	0.14		
Combined I, II, and III exposed to pH 3.2 and 23°C. for 18 hr.	50		0.8		.9	
Dialyzate	42	1.41	<0.8	<0.03	.8	<0.1

^a The yields are based on the original activity in each case.

^b The units/mg. TN for the original solution were 0.0012 for "cellulase" and 0.12 for amylase compared with 0.0025 for PE and 0.24 for PG.

Specificity Studies with Crude and Purified Polygalacturonase

The hydrolytic activities of Pectinols 45 AP and 46 AP were determined for 21 glycosidic substances. Tests were made under conditions that give good activity of PG on pectic acid. Admittedly, this procedure might fail to reveal the presence of potential enzyme activities that might become evident only under other conditions (*e. g.*, different pH values). On the other hand, it is unlikely that such activities would be due to PG, which, of course, is the enzyme of interest in this study.

Table VI shows that the crude pectic enzymes do not catalyze the hydrolysis of 9 substances related to pectin by virtue of their content of uronic acid or galactose and one unrelated substance, ovomucoid. Based on limits of error, pectic acid is hydrolyzed more than 10,000 times as fast as these substances. Table VII shows that essentially a similar situation exists for purified PG and starch, maltose, sucrose, the cellulose derivative carboxymethylcellulose, oxidized cellulose, inulin, and gum tragacanth. The small hydrolytic activity on pectin and the methyl glycoside of polygalacturonic acid methyl ester probably corresponds to the hydrolysis of those parts of the uronide chain that contain few or no ester groups or that were deesterified during the

TABLE VI
Substances Not Hydrolyzed by Crude Polygalacturonase

Substance	Approximate glycosidic composition ^a Percentages refer to anhydro residues	Substrate conc.	Relative activity ^b	
			46 AP	45 AP
Pectic acid (control)	α -D-galacturonic acid, 90% (13)	per cent 0.5	(100)	(100)
Methyl glycoside of galacturonic acid	Methyl α -D-galacturonide	1	<.009	<.003
Methyl glycoside of galacturonic acid methyl ester	Methyl α -D-galacturonide methyl ester	1	<.009	<.003
Ovomucoid	Mannose, 11%; glucose amine, 11% (14)	1	<.004	
Gum arabic	Galactose-glucuronic acid, 28%; hexose, 29%; pentose, 34%; methyl pentose, 14% (15)	1	<.004	<0.004
Gum ghatti	Galactose or galacturonic acid, 12%; pentosan, 50% (16)	1	<.002	<.007
Mesquite gum	L-arabinose, 51%; D-galactose, 31%; methyl-D-glucuronic acid, 18% (17)	1	<.004	<.007
Heparin	D-glucosamine, 22%; D-glucuronic acid, 22% (18)	0.5	<.003	
Pneumococcus polysaccharide (Type I)	D-galacturonic acid, 60% (19)	.25	<.004	
Alginic acid	β -D-Mannuronic acid, 95% (20,21)	.5	<.002	<.002
Hyaluronic acid	N-acetyl-D-glucuronic acid, 52%; glucuronic acid, 48% (22)	.25	<.005 ^c	

^a The percentage composition figures refer to the anhydro basis. The figures are frequently the authors' estimates based on the references cited. Although it has been necessary to make some assumptions to convert the data in the literature to a common basis, it is felt that the percentage figures given are sufficiently accurate for the present purpose.

^b The relative activities are expressed in terms of mM of CHO liberated/min./100 units of PG. Some of these substrates were tested with both purified and crude PG. No hydrolysis was observed in any case.

^c This figure is for purified PG. A test with the crude enzyme was not made in this case.

TABLE VII
*Relative Hydrolytic Activity of Crude and Purified Polygalacturonase
 on Glycosidic Substances*

Substance	Substrate conc.	Relative hydrolytic activity ^a			
		Pectinol 46 AP		Pectinol 45 AP	
		Crude	Purified	Crude	Purified
	<i>per cent</i>				
Pectic acid	0.5	100	100	100	100
The methyl glycoside of polygalacturonic acid	1	104	114	144	138
Starch	0.5	16.5	<.001	49.2	<.004
Pectin	0.5	6 ^b	.18	1.4 ^b	.026 ^b
Maltose	0.5	3	<.008	.47	<.005
Sucrose	0.5	1.9	<.004	.86	<.012
Cellulose derivative ^c	1	1.3	<.001	.52	<.019
Oxidized cellulose ^d	1	0.04	—	—	<.003
Inulin	1	.81	<.004	.08	<.006
The methyl glycoside of polygalacturonic acid methyl ester	1	.65 ^b	.04 ^b	.14	<.01
Gum tragacanth ^e	1	.12	<.001	.01	.012

^a See footnote b, Table VI.

^b Minimum figure. The rate decreases so rapidly with time that the initial rate could not be reliably measured. It is likely that the observed hydrolysis of pectin by PG corresponds to the hydrolysis of that part of the uronide chain that contains few or no methyl ester groups (6).

^c The sodium salt of carboxymethylcellulose (Dow Chemical Co.) was used for this test.

^d Oxidized cellulose gauze (Tennessee Eastman Corp.) was dissolved in dilute alkali and remained in solution when the pH was adjusted to 5.0 for assay. The oxidized cellulose was prepared with nitrogen dioxide. It contained approximately 20% carboxyl groups, which corresponds to about 80% oxidation of the primary hydroxyl groups.

^e Glycosidic components of gum tragacanth include arabinose, galacturonic acid, fucose and galactose.

reaction period by traces of PE (6). The tests with purified 45 AP and sucrose and carboxymethyl cellulose were shorter experiments; hence, the higher limits of activity. Although the activity of purified PG from 45 AP on gum tragacanth appeared to be real, it was, however, so low that it was not investigated further.

DISCUSSION

Since Jansen and MacDonnell (6) presented evidence to show that only one enzyme was involved in the hydrolysis of pectic acid and the successive intermediates to galacturonic acid, it appears that purified PG is heterospecific so far as molecular size of polygalacturonic acid substrate is concerned. However, since purified PG does not hydrolyze appreciably any of the 17 nonpectic polysaccharides and glycosides used in this study, it appears to be monospecific so far as kind of bond is concerned. Its failure to hydrolyze methyl α -D-galacturonic acid places it among the specific polysaccharidases (*e.g.*, α - and β -amylase) rather than among the simple glycosidases, which hydrolyze numerous glycosidic compounds that differ only in the aglycon part of the molecule. This is consistent with the results of Jansen and MacDonnell, which indicate that free carboxyl groups on two or more adjacent galacturonide residues are necessary for PG action (6).

The following types or classes of compounds are not hydrolyzed by PG, as indicated by its failure to hydrolyze the compounds named in parentheses following each class: (a) simple glycosides of galacturonic acid (methyl α -D-galacturonide); (b) glucuronic acid compounds (mesquite gum, heparin, oxidized cellulose, carboxymethylcellulose, and hyaluronic acid); (c) polymannuronic acid compounds (alginic acid); (d) glucose- and glucosamine-containing compounds (starch, sucrose, maltose, ovomucoid, and heparin); (e) fructose-containing compounds (sucrose and inulin); (f) galactose-containing compounds (mesquite gum and gum tragacanth, which was hydrolyzed only very slowly); and (g) pentose-containing compounds (gum ghatti, mesquite gum, gum arabic, and gum tragacanth).

It is obvious, of course, that the ring structure, the configuration of α , β linkage and presence or absence of substitution in the anhydro-residues as well as the kind of anhydro-residue may limit the action of PG on the various polymers studied. In this regard, the failure of PG to hydrolyze pneumococcus polysaccharide Type I is of particular interest since it contains 60% anhydro-D-galacturonic acid of the same configuration and ring structure as exists in pectin (19). Evidently linkages between galacturonic acid residues, if such linkages are present, are not the same as they are in pectic acid, or substitutions in the galacturonic acid residues occur and prevent PG action. Thus, purified PG should be, useful as an analytical tool to determine the occurrence in experimental

material (particularly glycosidic materials) of pectin-like galacturonic acid residues.

Purified PG would be useful also as an aid in removal of pectic acid and pectin (after deesterification) from mixtures of polyuronic acids and related substances. In some cases it might be necessary to facilitate PG action by deesterification with dilute alkali or with the other pectic enzyme, pectinesterase, which also is highly specific (23). The use of crude pectic enzymes in the analysis of feeds and food products was suggested by Isbell and Frush (24). They state that ". . . an analysis of dried beet pulp might include moisture, fat, protein, crude fiber, ash, and pectic-enzyme-soluble substances." The authors agree in principle with Isbell and Frush, but feel that the several enzyme activities found in the crude enzyme preparations make it desirable to determine pectic-enzyme soluble substances with purified enzymes. Extensive use of purified PG and PE for any of the above purposes will doubtless await commercial availability.

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SUMMARY AND CONCLUSIONS

Purified polygalacturonase (PG), like other polysaccharidases, was found to be highly specific. Based on sensitivity of the methods used, it hydrolyzes pectic acid at least 5000 times as fast as it does starch, maltose, sucrose, oxidized cellulose, carboxymethylcellulose, inulin, and gum tragacanth, which are hydrolyzed by crude polygalacturonase preparations. The remaining substances that were tested resisted hydrolysis by both crude and purified PG. They include gum arabic, mesquite gum, gum ghatti, ovomucoid, heparin, pneumococcus polysaccharide (Type I), methyl α -D-galacturonide, and hyaluronic acid. Failure to hydrolyze methyl α -D-galacturonide differentiates PG from

the simple glycosidases. Of the above-named substances, those that contain galacturonic acid residues evidently possess (a) different glycosidic linkages between residues or different α , β configuration than occurs in pectin or (b) if the links are the same (as they are in the case of the fully esterified portion of pectin, which is refractory to PG), then chemical substitution must prevent hydrolysis by PG.

The high degree of specificity of purified PG indicates its usefulness as a tool for detection and modification of pectic acids and pectins (after deesterification) that are present in other glycosidic substances.

The PG of Pectinol 45 AP was purified 7- to 10-fold by adsorption on alginic acid, elution, and dialysis. PG of Pectinol 46 AP was purified 20- to 30-fold by acid treatment, salt and lead acetate fractionation, and dialysis. The purified PGs from both sources had specific activities of about 1.7 units/mg. of nitrogen. Pectinesterase (PE) activity was removed during purification of 45 AP by acid treatment and was removed from 46 AP by a combination of acid treatment and salt fractionation. For preparation of PG essentially free of PE, these methods were more satisfactory than the method used by McColloch and Kertész in which the resin IR-100 is employed.

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The Influence of Sugar Content and pH on *In Vivo* Decalcification of Rat Molar Teeth by Acid Beverages

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INTRODUCTION

It has been previously reported (1,2,3,4,5) that a wide variety of acid beverages are capable of grossly eroding the molar teeth of white rats *in vivo*. Using a cola-type beverage containing 10% sucrose and 0.055% phosphoric acid, having a pH of about 2.6, Restarski and coworkers (2) observed that marked destruction of the lingual enamel resulted when rats consumed 20 ml. of this beverage daily for one week; when saccharin replaced the sucrose, the extent of destruction was considerably less even though the pH of the solution remained essentially the same. The conclusion was reached that sucrose somehow augmented the decalcifying properties of the phosphoric acid. Further studies (3) indicated that glucose and maltose behaved as did sucrose, whereas lactose had no such effect. With acids other than phosphoric (*i.e.*, lactic, citric, sulfuric) no difference in the extent of molar damage was noted (3) whether sucrose or saccharin was used as the sweetening agent. Accordingly, the present research was undertaken to ascertain the mechanism by which certain sugars apparently increased the tooth-destructive properties of phosphoric acid solutions.

PROCEDURE

Animals

Young albino rats (Sherman strain) 7-8 weeks of age and weighing 150 g. or more were used in all experiments. They were grouped comparably according to sex and litter and kept in separate cages so that the intake of fluid could be accurately controlled and recorded. All rats received commercial dog feed pellets *ad libitum* and 20 ml. daily of the appropriate test solution.

At the end of each 7-day experimental period the animals were sacrificed, their jaws removed, and the teeth examined and graded as previously described (6). According to this system of assessing tooth destruction, the individual upper and lower molars were given scores ranging from 0 (normal) to 6 (very severe destruction), depending on the extent of damage to the enamel and dentin.

Solutions

The concentrations of the sugars and the phosphoric acids in solutions fed to rats were the same as those previously employed by Gortner *et al.* (3), *i.e.*, 10% sugar and 0.055% phosphoric acid. In the sugar-free acid solutions, except in the initial experiment, 37.5 mg.-% of pure saccharin was added to make the solutions comparable in sweetness to the sucrose solution so that the rats would drink the full 20 ml. of solution given to them daily. When glucose or lactose was substituted for sucrose, saccharin was also added at the lower levels of 6.0 and 31.5 mg.-%, respectively.

In experiments concerning the *in vivo* decalcifying effects of phosphoric or lactic acid solutions of different pH, varying amounts of approximately 0.1 N NaOH were added by burette to 0.055% phosphoric acid-saccharin or 0.45% lactic acid-saccharin solutions to bring the initial pH (2.5–2.6) up to the desired level, as determined with a Beckman pH meter.

Glass drinking tubes having a J shape were used exclusively; by nature of their construction they permit maximum consumption of the solution with a negligible loss due to spillage and evaporation.

RESULTS AND DISCUSSION

To check earlier observations (2), an initial experiment was performed on the relative decalcifying effects of phosphoric acid-sucrose and phosphoric acid-saccharin solutions. To our surprise, typical etching of the molars resulted from the sugar-acid solution, but *no effect whatever* was noted with the saccharin-acid solution. On checking the pH of the two solutions, the former was found to be 2.5–2.6, while the latter was in excess of pH 4. The saccharin used in this experiment was a commercial type sold in tablet form; obviously something in the saccharin preparation had partially neutralized the acid and thus prevented tooth decalcification. This buffer substance proved to be sodium bicarbonate. The earlier studies of Restarski *et al.* (2) had also been carried out with a commercial saccharin preparation, but these workers had reported no significant change in the pH of their saccharin-containing solutions. Nevertheless, it appeared probable that an interfering substance of this type was responsible for the results which they observed. Likewise, the observation (3) that lactose behaved differently from sucrose, glucose, and maltose, might be explained on

the basis that a considerable amount of the saccharin preparation was added to the lactose solution to make it comparable in sweetness to the sucrose solution.

In all of our later studies chemically pure saccharin was used. Table I summarizes the results obtained when phosphoric acid solutions containing sucrose, glucose, lactose, and/or c.p. saccharin were consumed by rats for one week. It is clearly evident that all of the sugars behave similarly and that none of them has any significant influence on the extent of tooth decalcification by the acid.

TABLE I
In Vivo Decalcifying Effects of Phosphoric Acid-Sugar and Phosphoric Acid-Saccharin Solutions at pH 2.5-2.6 on Rat Molar Teeth

Solution	No. of rats	Average molar scores		
		Lower jaw	Upper jaw	Whole head
Acid-sucrose	13	3.7	2.7	3.2
Acid-lactose	6	3.6	2.8	3.2
Acid-glucose	4	3.8	3.0	3.4
Acid-saccharin	10	3.2	2.9	3.1
Sucrose (controls)	6	0.0	0.0	0.0

Two questions still presented themselves: (a) How can these results be reconciled with those of Restarski and coworkers (2), who noted no appreciable change in pH of their saccharin-containing solutions? (b) Why should the buffered saccharin tablets counter decalcification by phosphoric acid solutions but not that by other acids such as lactic and citric? The answers to both questions clearly lie in the titration curves of the various acids. Fig. 1 shows such curves obtained with phosphoric and lactic acids. It can be seen that with the former acid in the pH range of 2.3-3.0 there is little change in pH with a relatively large change in titratable acidity. Above pH 3.0, however, there is a large change in pH with only a small change in titratable acidity. As much sodium hydroxide is required to change the pH from 2.3 to 2.7 as is needed to change the pH from 2.7 to 6.2. Thus, although the saccharin preparation used by Restarski *et al.* (2) did not contribute enough buffer to materially increase the pH of their phosphoric acid solutions, it did apparently decrease the titratable acidity of such solutions to the

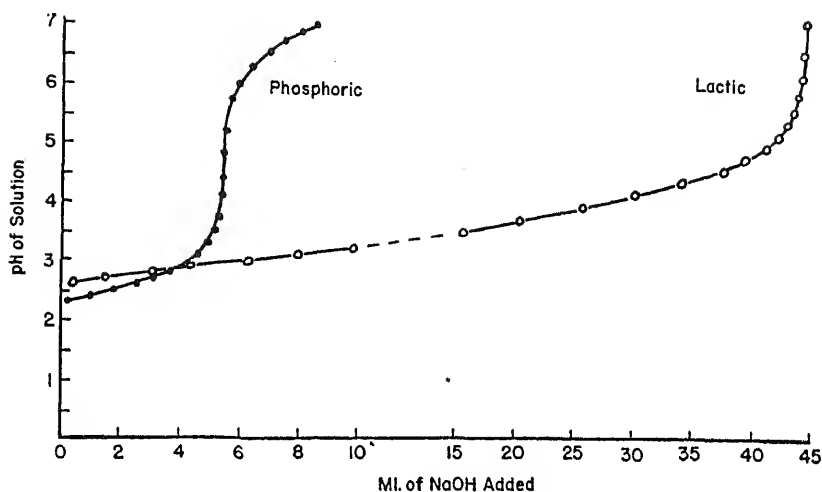


FIG. 1. Titration curves of phosphoric and lactic acids, showing the changes in pH accompanying the titration of (a) 50 ml. of 0.11% phosphoric acid and (b) 100 ml. of 0.45% lactic acid with 0.0987 *N* sodium hydroxide.

point where they no longer caused as severe tooth decalcification *in vivo*.

Lactic acid, being a weaker acid, requires a much greater concentration than phosphoric acid to give a solution of pH 2.5–2.6; consequently, far more base is needed to cause neutralization. The titration curve of lactic acid (Fig. 1) shows a gradual rise up to about pH 4.5, followed by a more rapid rise to the equivalence point, which is at a higher pH than that of phosphoric acid. It can be seen, therefore, that the small amount of buffer contained in commercial saccharin tablets would not appreciably modify the pH or titratable acidity of 0.45% lactic acid solutions. Thus, no differences in molar decalcification would be noted between rats receiving lactic acid-sucrose on the one hand, and lactic acid-commercial saccharin on the other.

On the basis of these titration curves, one would predict that, *in vivo*, a rapid decrease in enamel destruction would occur as the phosphoric acid solutions approached pH 3.0 and as the lactic acid solutions approached pH 4.5. This has been confirmed by feeding to rats phosphoric and lactic acid solutions of known pH, ranging from pH 2.5 to pH 4.5. The results, shown graphically in Fig. 2, demonstrate conclusively that the *titratable acidity* of the solution consumed, rather

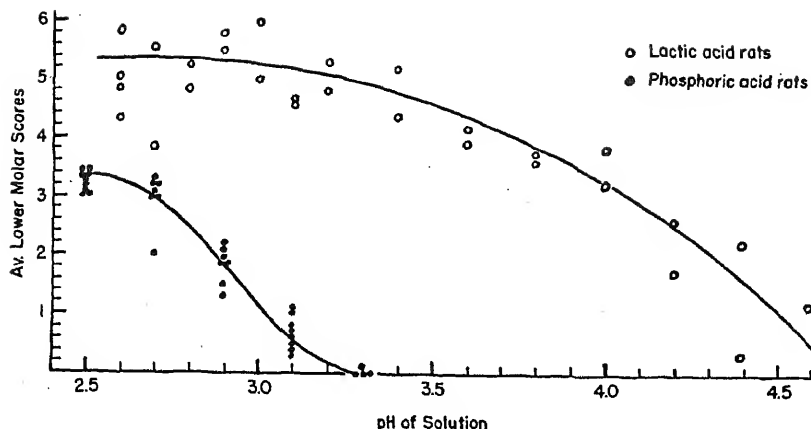


FIG. 2. The decalcifying effects of lactic acid and phosphoric acid solutions, containing saccharin, on the lower molars of albino rats. Each dot represents the average lower molar score for one rat.

than its pH, is the more important consideration in tooth decalcification. It is the final pH of the solution *in the mouth*, rather than the pH of the solution ingested, which determines the extent of enamel destruction; this, in turn, is a function of the buffering capacity of the saliva and of the properties characteristic of the acid under observation.

SUMMARY

1. The addition of 10% of sucrose, lactose, or glucose to a 0.055% phosphoric acid solution does not significantly alter the extent of decalcification, *in vivo*, of rat molars above or below that caused by a 0.055% phosphoric acid-saccharin solution.

2. The extent of enamel dissolution caused by phosphoric acid solutions *in vivo* drops rapidly when the pH of such solutions exceeds 2.7. At pH 3.3 no observable decalcification occurs during a period of one week.

3. The extent of *in vivo* tooth decalcification caused by lactic acid solutions remains fairly constant with solutions up to about pH 3.4, then decreases gradually until, at pH 4.6, enamel erosion is essentially absent.

4. The degree to which acid solutions attack the teeth is a function, not of the pH of the solutions ingested, but of the titratable acidity of such solutions and the buffering capacity of the saliva.

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LETTERS TO THE EDITORS

Metabolism of Myanesin (3-(*o*-Tolyoxy)-1,2-Propanediol)

It has been reported that animals receiving myanesin excrete little free drug, although appreciable quantities of a conjugated form appear in the urine (1,2). We have observed that experimental animals and humans also excrete in the urine, as early as 30 min. after administration of the drug, a substance which gives Ehrlich's diazo reaction.

We have isolated 2 closely associated organic acids from the urine of human patients with Parkinson's disease, following the administration of Myanesin. Neither one of these substances appears to be a drug conjugate. However, the second gives Ehrlich's diazo reaction. These compounds were concentrated by adsorption from the urine on Darco G 60, followed by elution with acetone. Subsequent chromatographic fractionation of cation-freed eluates on Doucil separated the two metabolic acids.

Suitable analytical methods for these two acids were not available at the start of this work and the quantitative aspects of the problem have not yet been investigated. It may suffice to indicate that partial collections of several days' urine of one patient receiving 3 g. of myanesin per day permitted the isolation of 1 g. of Compound 1 and 20 mg. of Compound 2.

Compound 1 was an optically inactive, relatively strong organic acid, with a neut. eq. of 198, and a $pK = 3.5$. It melted at $146^{\circ}C.$, subliming slowly at this temperature. Analysis showed C, 61.05; H, 5.82 and the absence of nitrogen and sulfur. Ultraviolet adsorption spectra indicated the likely presence of an *o*-cresol residue. It was considered plausible that Compound 1 was β -(*o*-tolyoxy)-lactic acid.

β -(*o*-tolyoxy)-lactic acid, which has not been previously described, was synthesized for comparison from sodium *o*-cresoxide and β -chlorolactic acid (3). The crude synthetic product, after charcoaling and recrystallization from hot water, was obtained as microcrystalline clusters of white needles. The compound melted at $146.5^{\circ}C.$ with slow sublima-

tion and gave no depression of melting point on admixture with Compound 1 isolated from human urine. Calc. for $C_{10}H_{12}O_4$; C, 61.20; H, 6.16; neut. eq., 196.1. Found: C, 61.37; H, 6.22; neut. eq., 194.3. β -(*o*-tolyoxy)-lactic acid is quite soluble in hot water, alcohol, acetone and ether. It is only slightly soluble in cold water. The molecular extinction, ϵ , at 2700\AA ($\epsilon = E \times \text{mol. wt.}/cd$), where E = extinction, C = conc. in g./l., and d = cell thickness in cm., was 1430 and 1390, respectively, for the isolated compound and for synthetic β -(*o*-tolyoxy)-lactic acid. Compound 1 appears to be identical with β -(*o*-tolyoxy)-lactic acid.

Compound 2, m.p. 168° , has not been obtained in sufficient quantity to permit certain identification, but work is continuing on this problem. The amounts of Compound 1 which may be isolated from the urine suggests that β -(*o*-tolyoxy)-lactic acid may be an important metabolic product of Myanesin. Compound 2 is excreted in small quantity and, from work already completed, it appears most probable that this compound represents a further step in the degradation of Myanesin,

It is noteworthy that β -(*o*-tolyoxy)-lactic acid was found to possess no Myanesin-like activity in the mouse.

Note added in proof: Since the submission of this Letter, a note has appeared by E. L. Graves, T. J. Elliott and W. Bradley, *Nature* **162**, 257 (Aug., 1948), which also announced the isolation of Compound 1, m.p. $146\text{--}147^\circ$.

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Note on the Electrophoretic Composition of Egg White¹

Recent electrophoretic analyses made in our laboratory are in disagreement with the contentions of Frampton and Romanoff (1). These workers concluded, on the basis of electrophoretic analysis, that there are at least 10 protein constituents in the white of the chicken egg, and that no major protein constituent in any one layer can be identified in the other layers. Their electrophoretic analyses were carried out at pH 4.45, at which pH we have found resolution to be difficult, and at which excessive protein interactions are possible. In addition, electrophoresis was carried out for such short periods of time, and under such low potential gradients, that the degree of resolution they obtained was too low to justify definite conclusions.

Conrad and Phillips (3), Conrad and Scott (4), and Burmester (2), have demonstrated that egg white is originally secreted as a "homogeneous protein gel" in the magnum and that formation of the inner and outer thin white is due to infusion of uterine fluids, coupled with the "mechanical segregation" of mucin fibers in the caudal portion of the oviduct. In accordance with these views and the probability of free diffusion of the soluble proteins in the white, major protein constituents would be expected to be common to all layers. A possible exception would be the "ovomucin" fraction which is responsible, presumably, for the structural characteristics of the thick layer.

The eggs used in this study were secured from the College Poultry Farm within 1 hr. after laying. They were immediately placed in the cold room (2-3°C.), broken, and the whites separated from the yolks. The three layers of the white were separated, homogenized, and the protein concentration of each adjusted to 1.0-1.5% by careful dilution with isotonic NaCl, no protein being lost by precipitation. Dialysis against the electrophoretic buffer was started within 4 hr. after laying and continued for 48 hr. The buffer used was phosphate with added NaCl, pH 7.8, total ionic strength, 0.2. Electrophoresis was continued for 360 min. under a potential gradient of 4.0-4.5 volts-cm.⁻¹.

Typical electrophoretic patterns for the layers are given in Fig. 1. These patterns should include the so-called ovomucin, but its identification has not been possible. Components are labeled in conformity

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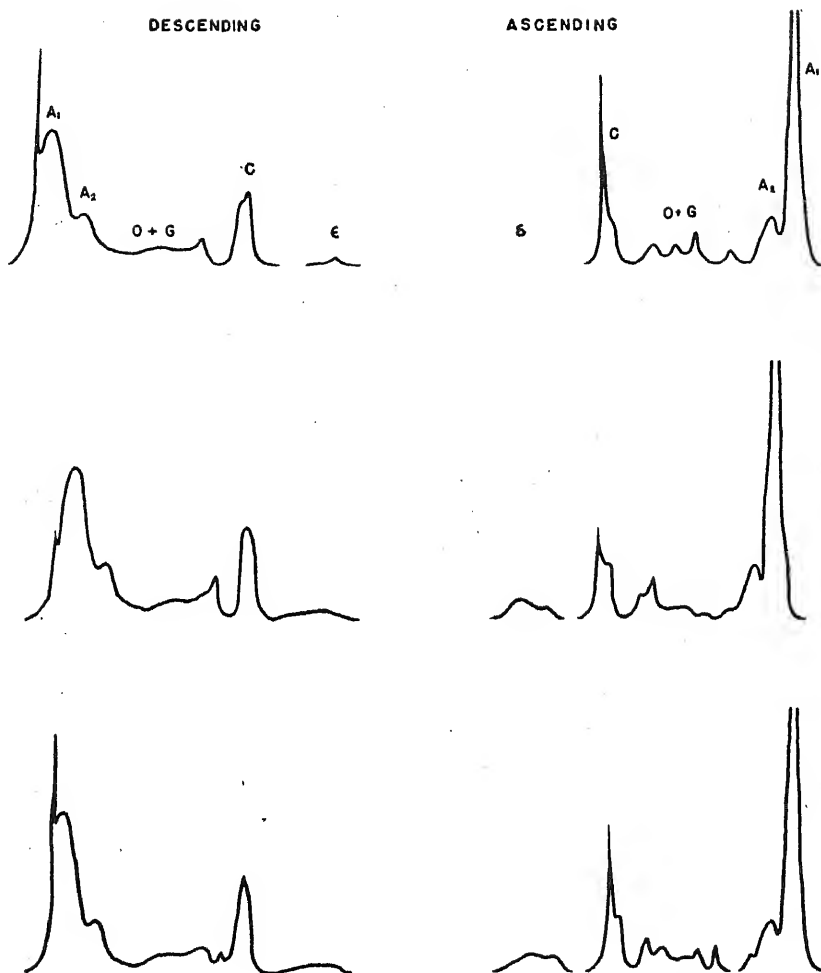


FIG. 1. Tracings of electrophoretic patterns of the 3 layers of egg white. From top to bottom, outer thin, thick, and inner thin. The region of the δ anomaly in the run on the outer thin layer was obscured in the negative.

with designations of Longworth *et al.* (5). Any differences noted between layers are within the limits of experimental error, with the possible exception of the sharp "spikes" on the ascending conalbumin and descending albumin peaks. Since they delimit the region in which both albumin and conalbumin are present, some interaction between

these components may be indicated. The effect appears to be greater in the thin layers. In Table I are summarized values of mobilities and relative concentrations for the more readily resolvable components. Data given represent averages of ascending and descending patterns obtained from 2 separate runs.

TABLE I
Mobilities and Relative Concentrations of the Components of Chicken Egg White

Component	Mobilities, $\text{cm.}^2\text{-volt}^{-1}\text{-sec.}^{-1} \times 10^5$			Relative concentration, per cent		
	Outer thin	Thick	Inner thin	Outer thin	Thick	Inner thin
A ₁	5.14	5.33	5.41	} 68.4	67.3	66.3
A ₂	4.66	4.89	4.88			
G ₃	2.66	2.45	2.65			
G ₂ , G ₃ , + 0	—	—	—	15.2	16.8	17.0
C	1.95	2.02	2.02	16.2	15.6	16.8

There would seem to be no justification for suggesting nonidentity of the protein of the 3 layers on the basis of electrophoretic measurements.

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The Effect of Pentobarbital on Aerobic Phosphorylation in Brain Homogenates ¹

There are several indications that drugs which reduce the activity of energy-requiring biologic processes may do so through interference

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with the formation or utilization of the energy-rich phosphate bonds made possible by the oxidation of substrate (1, 2, 3). Recently, Loomis and Lipmann (4) have shown, in preparations of rat kidney, that 2, 4-dinitrophenol inhibits the coupling between phosphorylation and oxidation. This same phenomenon has been observed in our rat brain homogenates. In marked contrast, however, to the effect of 2,4-dinitrophenol is the effect of pentobarbital.

TABLE I

To double-armed vessels was added 1.0 cc. of a 1-4 rat brain isotonic homogenate dialyzed^a for 2 hr. (2°C.) against 0.05 *M* phosphate buffer (pH 7.8). These extracts were similar to those of Ochoa (5). The following substances in micromoles were added: glucose 33, ATP 1.5, sodium fluoride 20 or 36, and magnesium chloride 15 (side arm). Water was added to make a total volume of 1.5 cc. Trichloroacetic acid was added to the remaining side arm. After 10 min. equilibration and gassing with oxygen, the magnesium was tipped in and identically prepared controls were stopped by tipping in the trichloroacetic acid. The experimental vessels were continued an additional 15 min., at which time the trichloroacetic acid was tipped in. If the oxygen uptakes measured at 5 min. intervals during this time were not constant, the run was discarded. Temperature 26.1°C.

Extract no.	Pentobarbital	Substrate added	Oxygen uptake	Phosphate uptake	P/O
	<i>molar</i>	<i>micromoles</i>	<i>microatoms</i>	<i>micromoles</i>	
37	0	Pyruvate ^b 21	14.6	22.5	1.5
	1.6×10^{-4}	Pyruvate ^b 21	12.8	20.3	1.6
	3.2×10^{-4}	Pyruvate ^b 21	10.1	17.6	1.7
40	0	Pyruvate ^b 21	12.7	24.4	1.9
	3.2×10^{-4}	Pyruvate ^b 21	10.0	19.8	2.0
	5.0×10^{-4}	Pyruvate ^b 21	7.0	11.3	1.6
	7.5×10^{-4}	Pyruvate ^b 21	1.9	2.0	1.1
36	1×10^{-2}	Pyruvate ^b 21	1.3	± 0	$\pm 0^c$
43	0	Succinate 20	8.7	12.0	1.4
	3.2×10^{-4}	Succinate 20	10.0	14.4	1.4
	5×10^{-4}	Succinate 20	9.9	13.7	1.4
	7.5×10^{-4}	Succinate 20	9.1	11.5	1.3
48	0	Fumarate 20	8.5	—	—
	7.5×10^{-4}	Fumarate 20	2.8	—	—

^a These extracts showed no phosphate uptake in the absence of pyruvate or succinate and no phosphate uptake was observed with pyruvate under anaerobic conditions.

^b Plus a trace of fumarate.

^c When the oxygen uptakes are corrected for this pentobarbital-stable respiration, the values for the P/O show no tendency to fall off.

The data in Table I indicate that pentobarbital inhibits the generation of high-energy phosphate bonds only to the extent that it interferes with the utilization of oxygen. With pyruvate as the substrate, pentobarbital inhibits both respiration and phosphate uptake proportionately, so that the P/O remains essentially constant in a given extract. With succinate as the substrate, neither oxygen utilization nor phosphate uptake is inhibited by the concentrations of pentobarbital studied. The P/O remains unchanged. Thus, not only does the oxidation of succinate escape the inhibition of the barbiturate, as pointed out by Jowett and Quastel (6), but the phosphorylation associated with this oxidation is left unaffected. The ability of succinate oxidation, under the conditions of our experiments, to generate high-energy bonds and thus furnish useful energy, encourages further studies (8) of the use of succinate as an analeptic in barbiturate poisoning.

According to Ochoa (7), there is probably only one phosphate bond associated with the oxidation of succinate to fumarate. The P/O of 1.4 observed with succinate in high concentrations of pentobarbital, together with the fact that this drug inhibits the oxidation of fumarate, appears to indicate that the transfer of a pair of electrons from succinate to oxygen *via* cytochrome c is associated with the generation of more than one high energy phosphate bond.

The data on other drugs, along with more complete studies on the use of pentobarbital as an agent useful in the study of the oxidative phosphorylation associated with succinate oxidation, will be presented in a future communication.

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Isolation of Biochemically Deficient Mutants of Bacteria by Limited Enrichment of the Medium

Lederberg and Tatum (1) have described a technic that greatly increases the efficiency of isolating bacterial mutants with increased growth requirements. It depends on selecting the unmutated cells by their ability to form large colonies on a minimal agar medium and the detection of the residue of mutants by the subsequent addition of a multiple supplement which will then allow the mutants to proliferate and be recognized as small colonies. As the proportion of detectable biochemical mutants in the viable bacterial population rarely exceeds 2%, even after optimal irradiation, this procedure obviously represents a major advance. Using this technic, we have isolated several biochemical mutants from ultraviolet irradiated *E. coli*. In our hands, however, a majority of the small colonies isolated were either normal organisms or "slow-growers" with no definite increased growth requirement. It is not known whether the normal organisms are products of the reversion of unstable mutants, as has been suggested (1), or have been delayed in starting colony formation for unknown physiological reasons. In any case, it would be desirable to eliminate their selection.

We have developed an alternative method of recognizing mutant colonies by using media with limited concentrations of the supplements. This technic is based on the observation that the bacteria studied are remarkably efficient in their capacity to extract growth factors from their environment. A tryptophan-requiring mutant, for example, was found to plate out as many colonies in a medium containing 0.001% enzymatic casein hydrolyzate as in a heavily enriched medium (0.2%), even though the colonies in the limited medium remained microscopic.

A minimal medium was employed containing ammonium lactate and glucose as sole sources of nitrogen and carbon, and sulfate as sole source of sulfur; this medium was enriched with small amounts of casein hydrolyzate, yeast extract, or pure growth factors. As it was not possible to predict accurately the concentration of a given factor which would yield microscopic colonies, several 5-fold dilutions of enriching factor were used. This dilution step was chosen because experience with previously isolated mutants had shown microscopic colonies to be formed over at least a 5-fold range of concentration. Several mutants requiring amino acids were found to produce the desired colony size

in concentrations of the magnitude of 1γ /ml. Vitamin-deficient mutants required much lower concentrations.

It has not been found necessary to employ several layers of agar, as recommended in the procedure of Lederberg and Tatum (1.) Instead, following irradiation, inocula containing 100 to 500 viable bacteria are poured on agar plates of media with limited enrichment. Troublesome formation of bubbles has been avoided by using thin layers of agar (10 ml. per Petri dish) and by incubating at temperatures somewhat below 37°C . Ordinarily, we have incubated at 37°C . for 24 hr. and then marked the unusually small colonies, recognized under a dissecting microscope. Following a second period of incubation for 24 hr., only the persistently small colonies are picked; many of the initially small ones have become large and are therefore eliminated. Under favorable circumstances, over 50% of the isolated colonies have proven to be stable deficient mutants.

With this procedure it has been possible to isolate, within a few weeks, mutants of ultraviolet irradiated *E. coli* ("Waksman" strain, ATCC No. 9637) with individual growth requirements for the following 12 amino acids: tryptophane, phenylalanine, tyrosine, leucine, histidine, lysine, arginine (or citrulline), arginine (or ornithine or citrulline), proline, threonine, glutamic acid, methionine, cystine (or methionine or sulfide), cystine (or methionine or sulfide or sulfite); for the following 4 vitamins: thiamine, pantothenic acid, niacinamide (or niacin), and pyridoxin (or pyridoxal or pyridoxamine); for the purines (adenine or guanine); and for unidentified constituents of yeast extract.

In addition to its value for isolation, limited enrichment permits accurate colony counts, in the same agar plate, of various mutants mixed with each other or with the stock strain. This procedure has proved useful in studies on mixed cultures.

Note added in proof: Since this manuscript was submitted, two reports have appeared in which limited enrichment was used for the recognition of biochemically deficient mutants (2, 3).

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Enzymatic Degradation of Triacetic Acid Lactone Determined by a Spectrophotometric Method

The high characteristic ultraviolet absorption of 2,4-diketo acids, and the use of this property in following the hydrolysis of these compounds by tissue homogenates (1), suggested the possibility that a spectrophotometric procedure might be of value in studying the intermediary metabolism of other polyketo acids. The enzymatic breakdown of triacetic acid lactone to acetoacetate, reported by Breusch and Ulusoy (2) in minced cat liver, has been studied by a spectrophotometric technique. Breusch and Ulusoy used the lactone, prepared as described by Collie (3), but refer to this compound as β,δ -diketohexanoic acid.

Triacetic acid lactone (TAL), the δ -lactone of 3,5-diketohexanoic acid (3), was found to exhibit maximum absorption at 285 and 275 $m\mu$ in 0.1 *N* HCl and 0.1 *N* NaOH, respectively (Fig. 1.) When TAL was incubated with rat liver homogenates at pH 7.4, the absorption determined in acid and in alkali decreased at all wavelengths. However, when incubated at pH 5.5–6.5, the spectrum determined in alkali shifted to a maximum at 295 $m\mu$, while the absorption curve in acid decreased progressively at all wavelengths. On further incubation the new spectrum disappeared slowly over a period of 2–3 hr. When the pH was adjusted to 7.0–7.4 in the presence of liver, disappearance of the new spectrum was complete in 30 min. Similar data were obtained under aerobic and anaerobic conditions. Incubation of TAL with kidney homogenates at pH 5.6 resulted in the disappearance of the characteristic absorption in acid of TAL, while the absorption determined in alkali increased considerably and the peak shifted to 295 $m\mu$ (Fig. 1). The rate of decrease of the absorption in acid was optimal when TAL was incubated at pH 5.8–6.0 and was about the same per g. of wet tissue in liver as in kidney. However, the disappearance of the new spectrum (in alkali) was most rapid at about pH 7 and was appreciably faster in liver. The absorption of TAL was unaltered by incubation with muscle or liver and kidney homogenates inactivated by standing at room temperature for several hr.

The evidence suggests that the unsaturated δ -lactone of 3,5-diketohexanoic acid is enzymatically converted to an intermediate compound possessing a spectrum similar to those of aliphatic 2,4-diketo acids (1) and acetylacetone (4) in that maximum absorption occurs at 295 $m\mu$

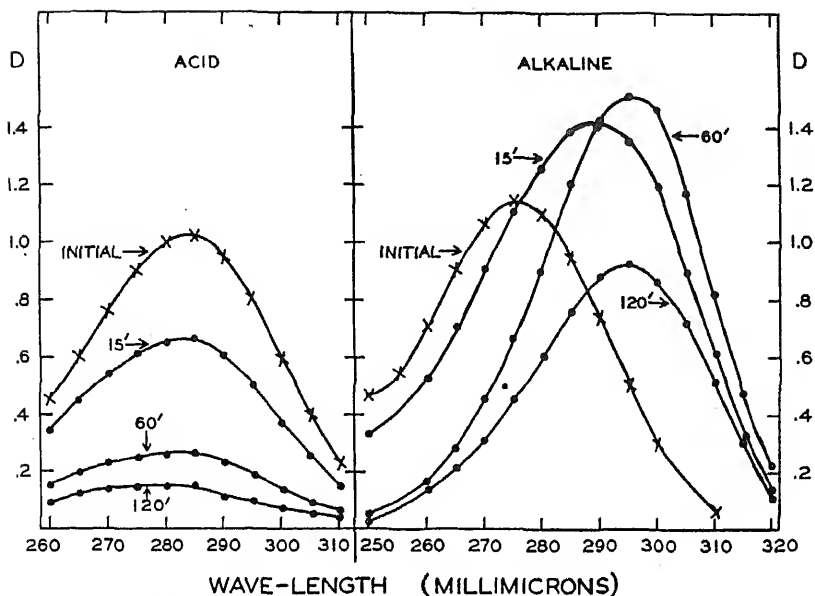


FIG. 1. Changes in absorption of triacetic acid lactone incubated with aqueous rat kidney homogenate. Equal volumes of homogenate (1 g. kidney to 2 cc. water) and triacetic acid lactone (0.025 *M*) in 0.08 *M* phosphate buffer were incubated at pH 5.6 and 37°C.; samples were removed at the indicated intervals, diluted with 99 volumes of 0.1 *N* HCl or NaOH and read against a homogenate blank in the Beckman DU spectrophotometer.

in alkali, and a reversible decrease of absorption occurs upon acidification. The data are compatible with the hypothesis that the intermediate is the free 3,5-diketohexanoic acid. This β -keto acid should be capable of readily decomposing to yield CO_2 and acetylacetone. Treatment with aniline-citrate (5) of a mixture of kidney homogenate and TAL after incubation for 1 hr. at pH 5.6 yielded 90–95% of the theoretical CO_2 . After acid distillation of the mixture, the distillate exhibited an absorption resembling that of acetylacetone and, when calculated on a quantitative basis, about 80% of the TAL metabolized could be accounted for as acetylacetone. The presence of the latter compound was further indicated by the formation of the characteristic red iron complex. Cf. (6). The hypothesis is supported by the finding that freshly prepared free 3,5-diketohexanoic acid (7,8) exhibited spectral and enzymatic properties similar to those of the intermediate.

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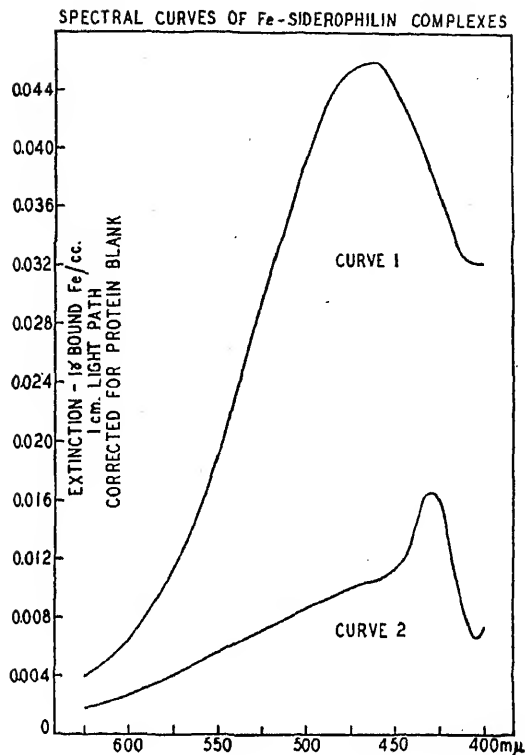
Carbon Dioxide and Oxygen in Complex Formation with Iron and Siderophilin, ¹ the Iron-Binding Component of Human Plasma (1,2,3)

Carbon dioxide (bicarbonate) is essential for full salmon-pink color development when ferrous iron is added under aerobic conditions to siderophilin² [$\epsilon_{1\text{ cm}}^{460\text{ m}\mu}$ (1 γ Fe⁺⁺/cc., pH 7.5) = 0.046 (Curve 1)]. The addition of increasing amounts of sodium bicarbonate to a decarbonated siderophilin solution, together with the requisite amount of Fe⁺⁺ (1 protein molecule:2 Fe atoms), leads to maximum color development when one molecule of sodium bicarbonate has been added per atom of iron. Once the full color has been developed, it is not diminished by equilibration of the complex with a CO₂-free gas phase.

Spectrophotometric measurements at 460 m μ of the iron-protein complex in the absence of added buffer, show that it begins to break up below pH 6.0, is 50% decomposed at pH ca. 4.8, and is completely broken up at pH ca. 4.0. The addition of increasing concentrations of phosphate buffer effects an increasing shift in these pH values toward

¹ This chemical designation, advanced several years ago and meaning literally iron-loving, avoids emphasis on any one demonstrated physiological function such as iron transfer or antibiosis.

² Siderophilin (β_1 -pseudoglobulin) was used either as fraction IV-7 or twice recrystallized fraction IV-7-4 (4). Both fractions were kindly supplied by Dr. E. J. Cohn and prepared by the Department of Physical Chemistry, Harvard Medical School, under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and Harvard University.



neutrality. Above neutrality, the affinity of the protein for the iron is very great, as is shown by a linear titration curve and a sharp break at saturation, even at a total siderophilin concentration as low as $3 \times 10^{-5} M$, the normal plasma level. At the normal plasma level of iron- CO_2 -siderophilin ($1 \times 10^{-5} M$), the CO_2 so bound corresponds to 0.02 vol.-% of the blood.

In the formation of the aerobic complex from ferrous iron and siderophilin, between 2 and 3 titratable H^+ ions are released for each atom of iron bound.

In the absence of oxygen, the addition of ferrous iron to the protein, together with excess sodium bicarbonate, leads to the development of a yellow colored complex [$\epsilon_{1\text{ cm.}}^{430\text{ m}\mu}$ ($1 \gamma \text{ Fe}^{++}/\text{cc.}$, pH 7.5) = 0.017 (Curve 2)]. However, when neutralized ascorbate (*ca.* $6 \times 10^{-3} M$) is present

with the protein under otherwise identical conditions, the salmon-pink complex characterized by the shape of Curve 1 is obtained, rather than the yellow anaerobic complex. It should be noted that the absolute value of $\epsilon_{1\text{ cm.}}^{460\text{ m}\mu}$ obtained anaerobically in the presence of ascorbate may be 0–25% less than that observed under aerobic conditions.

When conalbumin³ from egg white is substituted for siderophilin, similar results are obtained, with the exception that, anaerobically, in the absence of ascorbic acid, no absorption maximum is observed between $\lambda = 400\text{--}500\text{ m}\mu$. When monovalent or divalent copper instead of iron is used with siderophilin, bicarbonate is also required for maximum development of the characteristic absorption band at $\lambda = 435\text{ m}\mu$ [$\epsilon_{1\text{ cm.}}^{435\text{ m}\mu}$ ($1\text{ }\gamma\text{ Cu/cc.}$, pH 8) = 0.026] (5).

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New York, N. Y.
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- ARTHUR L. SCHADE
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On the Mode of Iron Binding by Siderophilin, Conalbumin, Hydroxylamine, Aspergillie Acid, and Other Hydroxamic Acids¹

In an attempt to elucidate the type of binding responsible for the formation of the specific, salmon-pink complexes of iron with conalbumin of egg white (1), or of iron with siderophilin (the iron-binding

³ The conalbumin used in this study was kindly supplied by Dr. H. L. Fevold of the Western Regional Research Laboratory, Albany, California.

¹ The siderophilin (plasma fraction IV-7) employed in this study was kindly supplied by Professor E. J. Cohn and Dr. Douglas Surgenor, Department of Physical Chemistry, Harvard Medical School, the conalbumin by Dr. H. L. Fevold, Western Regional Research Laboratory, and the aspergillie acid, hydroxyaspergillie acid, and related hydroxamic acid compounds by Drs. O. Wintersteiner, J. D. Dutcher and W. A. Lott of the Squibb Institute for Medical Research, respectively.

β_1 -pseudoglobulin of human plasma (2,3,4,5,6)), it has been found that aspergillic acid (N-hydroxy-3,6-di-*sec*-butylpyrazone-2) and hydroxylamine bind ferric iron under appropriate conditions to yield complexes with the same absorption spectrum maximum as shown by the two iron protein complexes, namely 460–465 $m\mu$, with the same order of extinction (0.025–0.05/cm. at 1 γ complex-Fe/ml.). An absorption maximum at approximately 460 $m\mu$ is also given by ferric hydroxyaspergillic acid and by ferric compounds of the simpler models N-hydroxy-4-methylpyridone-2, N-hydroxy-5-bromopyridone-2, and N-hydroxy-4,6-dimethylpyrimidone-2, all containing the cyclic hydroxamic acid grouping that Dutcher (7) has indicated to be responsible for iron binding by aspergillic acid.

Equally significant is the fact that carbon dioxide (bicarbonate) is required to form the salmon-pink complex with iron and hydroxylamine, just as Schade, Reinhart and Levy (6) have reported for formation of the salmon-pink complexes of siderophilin and conalbumin. On the other hand, with aspergillic acid and the related cyclic compounds listed, no carbon dioxide is required. These facts suggest that in the complexes with siderophilin, conalbumin, and hydroxylamine, carbon dioxide serves to form a primary or secondary hydroxamic acid type grouping equivalent to the carbonyl group adjacent to hydroxylated nitrogen already present in the cyclic compounds. In further agreement with the indicated iron-hydroxamic binding is the fact that the two proteins, like hydroxamic acids generally, yield colored complexes with copper also (6).²

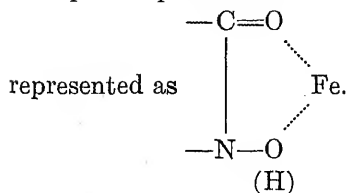
The measured stoichiometric ratio of Fe:C=O in the aspergillic acid complex with an absorption maximum at 460 $m\mu$ is 1:1, in harmony with the similar observation of Schade, Reinhart and Levy (6) that the stoichiometric ratio of Fe:CO₂ in the corresponding siderophilin complex is 1:1. The salmon-pink aspergillic acid complex is, however, stable only at quite acid pH values of less than about 4, as compared with the greater stability of the iron-CO₂ complexes of siderophilin, conalbumin, and hydroxylamine at neutrality.

² Of parallel biochemical interest are many early papers on the photochemical formation of formhydroxamic acid from nitrite and methyl alcohol or carbon dioxide (cf. O. Baudisch and collaborators, *Ber.* **44**, 1009 (1911) *Arch. Biochem.* **5**, 401 (1944) *et seq.*; E. C. C. Baly and collaborators, *Trans. Chem. Soc.*, **121**, 1078 (1922) *et seq.*; and D. Burk, *J. Phys. Chem.*, **31**, 1338 (1927) and *Dissertation*, Univ. of California, (1927)).

Aspergillic acid also forms a yellow iron complex that is stable at neutral as well as acid pH values, in which the ratio Fe:aspergillic acid is not 1:1 but 1:3, and the absorption spectrum maximum is shifted to 420 m μ . This yellow complex, in which protons have probably been dissociated from the hydroxyl groups ($pK = 5.3$), may coexist with the salmon-pink complex at acid pH values, the more so as the concentration of aspergillic acid is increased in relation to Fe. Hydroxylamine also yields a yellow (iron-CO₂) complex whose stability increases as the pH is raised above neutrality. Coexistence of two coordination complexes, cobaltodihistidine and cobaltomonohistidine, with greater stability of the former at higher pH values and higher histidine/Co concentration ratios, has also been observed (8).

Just as siderophilin, conalbumin, and hydroxylamine, in contrast to the C=O containing hydroxamic acids named, require CO₂ to complete their respective salmon-pink complexes, so siderophilin and conalbumin, in contrast to hydroxylamine and the hydroxamic acids (which contain already-formed OH groups), require OH⁻ for stabilization of their complexes, attaining virtual completion as the pH is raised to neutrality. The shape of the dissociation curve of the salmon-pink siderophilin complex, in the presence of buffers such as phosphate, citrate, or serum, indicates a loss of one hydroxyl group per atom of Fe given off (as tested with dipyriddy in the presence of hydrosulfite), with half dissociation at about pH 6.3, depending somewhat upon the concentration and kind of buffer. This stoichiometric relationship indicates a ratio of siderophilin to (Fe:CO₂:OH⁻) of 1:2(1:1:1). For the hydroxylamine and the hydroxamic acid compounds, where 1 instead of 2 atoms of Fe per molecule are involved, the corresponding ratio is 1:(1:1:1:), C=O being taken as equivalent to CO₂. Desoxy-aspergillic acid does not give the salmon-pink color with Fe.

The foregoing considerations thus indicate that the grouping in the siderophilin, conalbumin, and other related complexes yielding an absorption spectrum maximum in the region of 460 m μ might be



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The Effect of Substrates on the Endogenous Metabolism of Living Yeast

The extent of conversion of C^{14} -labeled "reserve" materials to CO_2 in yeast under various conditions has been examined by the following procedure. Suspensions of glucose-grown *S. cerevisiae* in $M/15$ KH_2PO_4 were allowed to assimilate radioactive glucose (anaerobically) or acetate (aerobically) until the substrate was exhausted, as indicated manometrically. The cells were then washed thoroughly, resuspended in buffer, and exposed to (a) no substrate, (b) inactive glucose, or (c) inactive acetate, in Warburg vessels containing KOH in the center well. After 75 min. at $30^\circ C.$, the experiment was ended and the metabolic CO_2 recovered from the KOH by precipitation as $BaCO_3$. C^{14} activity was estimated with a Geiger-Mueller counter as described by Kamen (1).

Typical results showing the effect of utilizable substrates on the appearance of labeled carbon (from reserves) in the metabolic CO_2 are given in Tables I and II.

In control experiments, fractionation of the cells just after the end of the initial assimilation disclosed a relatively high level of C^{14} in the acid-soluble portion. In this connection, it should be recalled that Fales and Baumberger (2) have recently demonstrated the appearance of an appreciable fraction of the carbon assimilated from glucose in an

TABLE I

Total and Specific Activities of Metabolic CO₂ from Labeled Cells^a

Exp.	C ¹⁴ , c/m/mg. C ¹²	C ¹⁴ , total c/m
1. Endogenous	1960	880
2. Exogenous (glucose)	880	3200

^a After fermentation of radioglucose, the cells were allowed to remain under N₂ for 2 hr. before use. The experiment was done under anaerobic conditions.

TABLE II

Total Activities of Metabolic CO₂ from Labeled Cells Under Various Conditions ^a

Exp.	Assimilated substrate	Inactive substrate added	C ¹⁴ in CO ₂ , total c/m
A.	C ¹⁴ -glucose	0	130
		glucose	270
B.	C ¹⁴ -glucose	0	430
		glucose	750
C.	C ¹⁴ -acetate	0	1530
		acetate	3300
		glucose	2800

^a In all cases, the conditions were aerobic during oxidation with or without inactive substrate.

acid-soluble form. In the present experiments, this was true in the assimilation of both radioactive acetate and glucose. When labeled glucose was used for the assimilation, it was found that the activity in the soluble fraction could be markedly diminished by allowing the yeast to remain under N₂ for 1–2 hr. after the fermentation of radioactive glucose had ceased. This treatment also results in a striking increase of C¹⁴ in the lipid fraction. Preliminary experiments, in which the relative specific activities of various cellular fractions were measured, indicate that, in yeast labeled by this procedure, the endogenous metabolic CO₂ is derived primarily from the lipid fraction. When glucose is furnished to such yeast, the lipid apparently still provides most of the metabolic C¹⁴O₂ observed.

From the foregoing data, it appears that "endogenous" metabolism in yeast is accelerated by the presence of substrate. However, the existence of a pool of metabolic intermediates common to both "endogenous" and "exogenous" metabolism and the obvious ease of exchange between various cell fractions make it evident that the manometric correction to be used in assimilation experiments cannot be obtained directly from data on isotopic exchange.

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Book Reviews

Advances in Protein Chemistry, Vol. IV. Edited by M. L. ANSON and JOHN T. EDSALL. Academic Press, Inc., New York, N. Y., 1948. 575 + ix pp. Price \$8.50

Volume IV of this valuable series contains 8 reviews, all excellent, but two of outstanding importance. Wyman's essay on the Heme Proteins is designed, as the author states in his summary, "to bring out the beautiful interdependence of structure and function in the heme proteins, and, in particular, the balance between the character of the heme, the character of the protein, and the nature of the heme-protein linkage, in determining the properties of the molecule." Including 6 pages of bibliography, this essay is 121 pages long and is the most thorough, most scholarly, and most informative treatment of this fundamentally important group of substances yet to appear. Wyman devotes the first 25 pages of the paper to a description of the properties of the heme proteins. The most accurate available physical and chemical data are collected and appraised and the main implications of the measurements are set forth.

He then turns to the development of entirely general equations to describe the interdependence of the chemical functions in the case of molecules that possess two or more different functions. These equations describe the linkage, in the case of hemoglobin, for example, between the oxygen-combining group and the proton-dissociating or acid group or groups, the behavior of which is in combination accountable for the well-known Bohr effect. The equations are applied to the study of oxygen equilibria of hemoglobin and lead to the conclusion that the 4 oxygen-combining centers, the heme groups, are inherently indistinguishable but are subject to interactions such that oxygenation becomes easier as it proceeds. The 4 hemes occur in identical pairs, members of the same pair interacting very strongly, members of different pairs much less strongly.

The implications of the Bohr effect, the observation that changes in partial pressure of the carbon dioxide influence the oxygen equilibrium, are fully discussed in terms of the linkage between the oxygen-combining and the acid groups, the interpretation being given mathematical form. The acid groups are identified from the heats of dissociation as the imidazole group of histidine, in the case of the weaker one, and as, probably, another imidazole group in the case of the stronger one.

The equilibria that involve oxygen and carbon monoxide are also subjected to analysis, and Haldane's 3 laws are shown mathematically to be expressions of a common mechanism and to be derivable from each other. There is a critique of the role of carbamino compounds in the elimination of carbon dioxide and the conclusion is reached that this role is by no means so large as has been suggested in the past, the oxygen Bohr effect being accountable for all but about 10% of carbon dioxide transport in the blood. Finally, there is a clear discussion of the magnetic properties of hemoglobin and of the oxidation-reduction phenomena of the heme proteins. To the extent that is possible, the conclusions are applied to the heme proteins other than hemoglobin.

One of the most significant conclusions of the paper is that the molecule of hemoglobin is a flattened disc of approximately the figure of a right cylinder 34 Å high and 57 Å in diameter, probably with somewhat bulging ends. It possesses a two-fold axis of symmetry perpendicular to the cylinder axis, and appears to consist of 4 equally spaced layers of matter. The hemes are arranged on the surface of the molecule with their planes parallel to the cylinder axis and the axis of symmetry. Each heme is united to an identical configuration of the globin so that they are inherently all alike but are grouped in identical pairs which have stabilizing interactions between members of each pair. This physical picture of the hemoglobin molecule is perhaps the most circumstantial account of the gross structure of a protein molecule yet to be given.

The other outstanding paper in Volume IV is that by Neuberger on the Stereochemistry of Amino Acids. This discussion is especially welcome at the present time when new practices in the nomenclature of amino acids, practices founded on the configurational relationships of the amino acids, are coming into common use. The author discusses, first, the evidence for the statement that the isomers of the amino acids which are commonly found in proteins all belong to the L_s family. He concludes that there can be no doubt that these isomers are, indeed, configurationally related to each other; however, there is no one line of evidence that, taken by itself, is completely convincing. The cumulative weight of the evidence from many different sources, and its consistent character, nevertheless, leave no reasonable doubt that the hypothesis of L configuration is correct.

The second section of Neuberger's review deals with the reactions of amino acids which involve substitution on the α -carbon atom, and includes a helpful treatment of Walden inversion. The third section takes up racemization, its mechanism, and the special techniques that have been devised to effect racemization. The last section discusses the D-amino acids and their occurrence in nature, and includes some interesting speculation on the possibility that the cyclic peptides, such as gramicidin, are more readily formed if amino acids of both configurations are present. Each section is terminated with a brief summary of its contents, an admirable feature in reviews of this type.

The other papers in Volume IV are addressed to specialists within the field of protein chemistry and deal with the several topics from a less general point of view. J. D. Ferry has contributed a chapter on Protein Gels, 78 pp. in length, in which the different types of gel formation are discussed. The entire field is carefully organized into gels that arise from the action of long-range forces, such as those of tobacco mosaic virus, network gels formed by the association at widely separated points of long chain molecules, such as that of gelatin, gels of denatured protein which apparently owe their specific properties to the unfolding of polypeptide chains and the association of these chains at any point along their lengths rather than at widely separated foci. Lastly, the author discusses the types of gel formed by blood fibrin to which he has contributed so extensively during recent years. In the structure of the fibrin gel, both types of association seem to be involved.

F. W. Putnam's chapter on the Interaction of Proteins and Synthetic Detergents collects a great deal of scattered information in this highly specialized field into a useful and brief summary. A. M. Pappenheimer, Jr., contributes a short but thorough

statement regarding the Proteins of Pathogenic Bacteria. A. B. Gutman has collected the available information regarding the Plasma Proteins in Disease in an article that runs to 85 pp. H. Svensson summarizes the methods for Preparative Electrophoresis and Ionophoresis, and has furnished helpful illustrations of the more important kinds of apparatus for the various procedures that have been described. R. B. Corey has compressed into 21 pp. the main accomplishments of specialists on X-Ray Studies of Amino Acids and Peptides.

Taken together, this group of articles on the several specialties of protein chemistry is highly informative and most useful. The papers of Wyman and of Neuberger will undoubtedly be required reading in graduate schools for many years. In view of this, the high price of the volume is most unfortunate, for it will operate to prevent, among students of protein chemistry, the wide circulation that is much to be desired. The Editors and Publishers should take seriously into consideration any possible change in policy that would serve to make such valuable contributions to the literature of proteins more readily available.

H. B. VICKERY, New Haven, Conn.

Advances in Biological and Medical Physics. Vol. I. Ed. by J. H. LAWRENCE AND J. G. HAMILTON, Academic Press, Inc., New York, 1948. 484 + xi pp. Price \$8.60.

This volume is the first in a projected series to be published yearly surveying progress in three categories; (1) radiation research, (2) radiochemistry, and (3) tracer methods in biology and medicine. The editors have chosen to devote almost all of this first volume to the third category.

In the last two years there have been published the proceedings of more than a dozen symposia on tracer research and its ramifications in biology and medicine. It is not surprising that most of the material in this most recent of "Advances" is available elsewhere in the literature. Despite this, there is much to recommend in the present publication.

Ten articles are included dealing with the following subjects: carbon and nitrogen isotopes, metabolism of blood, phospholipides, iodine, nucleic acids; nature and production of artificial radioactivity; fundamentals of radioactivity and radioactivity assay instrumentation; use of isotopes in medicine; irradiation effects in the wake of the atomic bomb; and health physics. Some will cavil with this scattering of subject matter; others will consider such variety no objection. To the reviewer it seems likely and desirable that organization of subject matter based on the tracer method as a thing-in-itself will be noted with less and less frequency in the future.

The wide scope of the material to be reviewed obviates any attempt to indicate the content of this monograph. It is to be expected that the relative value of the various articles will depend on the predilection of the reader. The reviewer was impressed particularly by Professor Vennesland's treatment of studies with nitrogen and carbon isotopes. Others with different interests will react similarly to the many other excellent articles, the quality of which is attested by names such as Hevesy, Evans, Chaikoff, and others.

The typography and format are excellent.

MARTIN D. KAMEN, St. Louis, Missouri

Recent Progress in Hormone Research, Vol. II. Edited by GREGORY PINCUS, Academic Press, Inc., New York, N. Y., 1948. v + 427 pp. Price \$8.00.

This volume contains the papers given at the 1946 Laurentian Hormone Conference together with the ensuing discussions, in the same way that Vol. I recorded the proceedings of the 1945 Conference. It can be guessed from the preface to Volume I that the attractions of the Laurentian Hormone Conferences are not entirely hormonal, but the proceedings of the 1946 Conference, like those of the preceding one, seem to have been more than adequately substantial to warrant a visit to one of the world's beauty spots.

The present volume contains 14 papers, 2 on physical methods, 3 on pituitary control, 2 on hormone metabolism, 4 on hormonal regulation of metabolism, and 3 on clinical endocrinology. Of these 5 main subjects only the last two were featured in the previous volume. Whether this fact be due to accident or design, the continued prominence of metabolic and clinical papers is of interest in considering past and future trends in hormone research. Endocrinology, as we now know it, is largely a product of the 21 years between the two wars. The hormones discovered before 1918, with one exception, were those which could be investigated in the acute experiments traditional to mammalian physiology. This group included adrenalin, secretin, and the posterior lobe principles. The exception, thyroid hormone, was particularly open to investigation because of being active orally. Even so, the synthesis of the active component was carried out between the wars. All the other chronically-acting hormones were discovered in the 1918-1939 period—insulin, parathormone, at least 3 gonadal hormones, several active cortical principles, 4 gonadotrophins of hypophyseal or other origin, and several other anterior pituitary hormones. Moreover, the more simple of these hormones have now all been isolated and prepared artificially. This spate of discovery must have carried away most of the nuggets of endocrinology; it is difficult to see how the endocrine organs, as we now know them, can in future yield up treasures of discovery as prolifically as they did between the wars. Extension of the era of great discoveries will depend on an extension of the classical concept of an endocrine organ. In what way, therefore, will endocrinology as we now know it travel in the near future? The most important of the major contributions waiting to be made are probably in connection with the chemical nature, identity, and relationship of the anterior hypophyseal hormones and their analogs found in body fluids. This work is likely to be prolonged and tedious if the history of other hormone work involving protein complexes is any guide. More immediate prospects lie in the sphere of general consolidation. The era of discovery left a multitude of untidy ends and loose bricks lying about, and it is evident that a phase of tidying up has been entered upon. It is in this direction that the Laurentian Conference is playing and will play such a useful part, and it is good to see that the emphasis at both of the last two conferences has been on the two largest piles of loose bricks—metabolic and clinical aspects of endocrinology.

A. S. PARKES, London, England

The Collected Papers of C. S. Hudson, Vol. II. Edited by R. M. HANN and N. K. RICHTMYER, National Institute of Health, U. S. Public Health Service, Bethesda, Maryland. Academic Press, Inc., New York, 1948. Price \$15.00.

A year ago the first volume of the *Collected Papers of C. S. Hudson* was published and its first copy presented to Claude Silbert Hudson at the festivities connected

with his 65th birthday. Now the second volume has been published and, like the first, it is edited by Hann and Richtymer of the National Institute of Health, Bethesda, Maryland.

Substantial contributions have been made by the Sugar Research Foundation, the Corn Industries Research Foundation, and the Coca Cola Company, to make publication possible. Just as the use of sugar encompasses the entire globe, and the Sugar Research Foundation, under the direction of its scientific director, Prof. Robert C. Hockett, engages in a comprehensive program of research, the masterly investigations of Hudson and his coworkers extend to the entire field of sugar chemistry. In view of the admirable versatility of subject matter treated by Hudson, any detailed review would necessitate a report on modern carbohydrate chemistry if it were to refer to all of the 128 publications included in Vol. II. The fundamental results have become part of present day teaching material. It should, however, be pointed out that an orientation in the chemistry of ketoses, particularly ketoheptoses, is greatly facilitated by a perusal of this volume, and this also applies to higher carbon sugars. The reader will realize with admiration how periodate oxidation in the sugar group has led to valuable new results and how Hudson has rejuvenated an apparently closed chapter of sugar chemistry—the osazones—by transforming them into osotriazoles by a simple treatment with copper sulfate. Due to the characteristic properties of the resulting substances this reaction has become an important analytical method.

All who know Hudson feel that his life's work is by no means concluded with the 247 publications thus far existent in the form of these collective volumes.

C. NEUBERG, New York

Newer Methods of Preparative Organic Chemistry. A compilation of a series of articles by German scientists. Published and distributed in the public interest with the consent of the alien property custodian under license No. A-1253. Interscience Publishers, Inc., New York, N. Y. xiii + 657 pp. Price \$8.50.

Beginning in 1940, a series of articles on methods of preparative organic chemistry appeared in *Die Chemie* and in 1942 were assembled in book form. Each was written by a distinguished German scientist, a specialist in the subject covered. The present book comprises 14 of these articles translated, revised, and partly brought up to date by a group of American chemists.

The articles which appear as chapters in this book are as follows:

Oxidations with Lead Tetraacetate and Periodic Acid, by R. Criegee; Dehydrogenation with Sulfur, Selenium, and Platinum Metals, by P. A. Plattner; Reduction with Raney Nickel Catalysts, by R. Schröter; Hydrogenation with Copper Chromite Catalysts, by Ch. Grundmann; Meerwein-Ponndorf Reduction and Oppenauer Oxidation, by T. Bersin; The Use of Biochemical Oxidations and Reductions for Preparative Purposes, by F. G. Fischer; Substitution Reactions of Aliphatic Compounds, by J. Nelles; Organic Fluorine Compounds, by W. Bockemüller; Catalysis of Organic Reactions by Boron Fluoride, by D. Kastner; Use of Hydrogen Fluoride in Organic Reactions, by K. Wiechert; Methods for the Thiocyanation of Organic Compounds, by H. P. Kaufmann; The Diene Syntheses, by K. Alder; Syntheses with Diazomethane, by B. Eistert; and Syntheses with Organolithium Compounds, by G. Wittig.

The American chemists who translated and revised the chapters are E. C. Armstrong, D. M. Burness, C. O. Edens, Jr., Bruce Graham, J. E. Jones, C. J. Kibler, I. Salminen, F. W. Spangler, J. R. Thirtle, J. A. VanAllan, C. V. Wilson, G. B. Bachman, Jean V. Crawford, R. J. Tull, Eleanor R. Webster, D. R. Goddard, and K. G. Stern.

No uniform style is found in the chapters. Each subject has been presented in the form which the author believes will be most useful to the reader. Usually a brief historical survey, and sometimes a short discussion of mechanism, precede sections dealing with each important application of the reagent or reaction. Since most of the subjects have been extensively studied and are of wide application, the authors have, of necessity, condensed their discussion to a minimum. In spite of the limited number of pages devoted to each subject, the important applications in each instance appear to have been covered. Thus, the reader can acquire in a very short reading time a review of the field and, through the use of the liberal supply of references, find readily the source of further information on any particular item of interest. Many of the chapters contain experimental directions for carrying out typical reactions.

A very comprehensive compound index has been compiled which includes the name of any substance mentioned in the chapters, no matter how casually. Along with an effective subject index, the reader is provided with a convenient means of locating with facility any part of the subject matter in the book. References to *Chem. Abstracts* have been substituted for those to *Chem. Zentr.*

One of the greatest problems for the organic chemist today is to know how to keep abreast of the voluminous literature which is now appearing in the field. A partial answer to this difficulty is the assembling of reviews of various subjects no matter how broad or narrow they may be. This book serves admirably to fill the need in the chosen areas. Even though some of the subjects reviewed in chapters in this book are to be found reviewed and summarized elsewhere, sometimes in more complete form, a different presentation of the same subject by another author has its value to the research man. This book supplements and extends the reviews of special topics in organic chemistry.

The book is well printed and contains a minimum of typographical and other errors. The translations are, in general, entirely satisfactory. The research man and the advanced student in organic chemistry may well add this volume to his collection of reference works.

ROGER ADAMS, Urbana, Ill.

Quantitative Organische Mikroanalyse. 5th ed. By F. PREGL, edited by H. ROTH. Springer-Verlag, Vienna, 1947. 317 pp., 80 figs. Price \$7.40 (Sw. fr. 32).

This latest in the series of texts on Pregl's methods for organic micro analysis, comprises sections on balances (15 pp.), determinations of elements (175 pp.), determinations of structural groups (79 pp.), and determinations of physical constants (39 pp.).

To this reviewer, the text was disappointing, inasmuch as many of the methods in common use in this country are omitted, or dismissed with a mere mention. In the section on balances, there is no mention made of the use of semimicro or macro analytical balances in micro procedures. The author apparently does not deem the Poth CO₂ generator worthy of discussion, although this reviewer has consistently obtained no

readable blanks with this type of generator. In like manner, dry ice as a source of CO_2 is dismissed with a one-page discussion of the Dewar flask, nothing at all being said about the use of a gasometer for long-term storage. In considering S and halogen determinations, the use of the Parr micro bomb is dismissed with 5 lines, although this is a standard method for determining both of these substances when present in solids or non-volatile liquids. Neither is there any mention made of the titrimetric method for sulfates using THQ, another standard method for busy laboratories. There are no references to the use of automatic combustion furnaces, and methods for the determination of molecular weights by boiling point elevation are eliminated.

The references seem very incomplete and do not run beyond 1943. In addition, of the 351 references cited in the section on elementary analysis, only 20 are to British or American publications; all others are to continental European, chiefly German or Austrian journals.

The substance "Drierite" (J. T. Baker) is listed as "deritrite of I. Baker." There is no author index, no log table, and no table for nitrogen reduction, the last a very serious omission, although the author infers, and in some cases specifies, that Küster's "Rechentafeln" are to be used.

However, the criticisms presented here should be considered against the background of the lack of available facilities, both library and laboratory, during the intra- and postbellum periods. Keeping these conditions in mind, the text is of the same value, especially to beginners, as the original Pregl texts.

W. A. HYNES, New York, N. Y.

Bioquímica Analítica Cuantitativa. By AGUSTIN D. MARENZI, CARLOS E. CARDINI, ROBERTO F. BANFI and FREDERICO A. S. VILALLONGA. Libreria y Editorial "El Ateneo," Buenos Aires, Argentina. Price \$60.—m/n.

In 50 chapters, comprising 1198 pages, this book treats of the methods of quantitative biochemical analysis in animal biochemistry. Essentially this is a handbook for clinical chemical investigations. Thus, stress has first and foremost been laid on methods required for this purpose. They have been selected expertly and described in considerable detail. Physicochemical methods are well represented. Each chapter is preceded by a historical introduction, which for obvious reasons has been dictated by the subjective views of the authors. The newest methodological results have been considered with insight and understanding for the needs of the users. It would seem desirable, however, to include a chapter on polarimetry and Warburg's manometric technique in a new edition of this remarkable book. The authors' own great experience gives it a personal note and may be deduced from their critical treatment of the material.

C. NEUBERG, New York, N. Y.

Remarkable Properties of Nucleic Acids and Nucleotides

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It is common knowledge that nucleotides are involved in many enzyme processes. Nucleic acids seem to participate in nearly all cellular activities. Apparently, there is hardly a single biochemical phenomenon the occurrence or course of which has not, in one way or another, been connected with nucleic acids. Two reviews by Tipson (1) and two symposia (2,3) provide a cross section of the vast amount of literature in this field. These reviews also specify the fundamentals established in certain special branches and the theories and assumptions prevalent in other fields.

With regard to the reactions of nucleic acids with substances encountered in the cells, or with which the nucleic acids are brought in contact during the course of cellular metabolism, the combinations with proteins were considered particularly. There are indications that in the cellular nuclei the nucleic acids may actually be linked also to inorganic cations. Despite this fact, the *reactions of the nucleic acids with inorganic substances* have not been thoroughly investigated. Some aspects of this problem will be described in this paper in view of the increasingly recognized significance of mineral substances in all biochemical processes.

The capability of the nucleic acids and nucleotides to form salts has always been used to isolate and characterize the corresponding acids. Whereas the alkali salts are soluble, the salts with bivalent or higher valent cations are mostly much less soluble or insoluble.

During the past 60 years of investigation this has been established for the nucleates of the alkaline earths, of Zn, Fe, Cu, La, Ag, Hg, Pb, and UO_2 . The corresponding data are found, *e.g.*, in papers by Altmann, Ascoli, Bang, Davidson and Weymouth, v. Euler, Feulgen, Greenstein, Hammarsten, Jackson, Jorpes, Levene and Jacobs, Liesegang, Neuberg, Osborne and Harris, Peiser, Schmiedeberg, G. Schmidt, Steudel, Takahata, Thanuhauser and Tipson. Both types of nucleic acids, the ribo- and the desoxyribonucleates, are characterized by their precipitability by metallic ions.

Despite the fact that no essential differences have been described for the two different types of nucleic acids, such differences do exist in individual instances, for differences in the acidity of these nucleic acids have been claimed, and the rates of polymerization exert a definite effect.

The nucleic acids are frequently separated from other cellular components by means of alkali. The resulting alkaline solution is neutralized and subsequently precipitated by means of suitable metallic salts. We have not found any description of the phenomenon that the insoluble nucleates obtained according to the above procedure are soluble in water-soluble nucleates such as the alkali nucleates. In the following it will be shown that this characteristic behavior is of fairly general validity. This fact should be duly considered. In connection therewith, it was found that bivalent or polyvalent metals at adequate excess of nucleate show entirely abnormal reactions. The precipitability of these metals as phosphates, arsenates, borates, carbonates, silicates, fluorides, sulfides, sulfites, sulfates, chromates, and molybdates is prohibited, and other types of ionic reactions are prevented.

The solubility of these metal-nucleic acid compounds in sodium nucleate is distinct throughout and, in some cases, substantial, although not always obvious on initial observation. This also seems to account for the fact that this phenomenon has not been observed by the authors of the respective papers concerned with methods of preparation.

The rather prevalent fact that many nucleates with bi- or higher valent cations are soluble in alkali nucleates may have escaped observation because the problem chiefly consisted in finding a method for the quantitative separation of the nucleic acids. Hence, neither the nucleic acids nor the nucleotides, but rather the precipitants, such as CaCl_2 , FeCl_3 , or $\text{UO}_2(\text{NO}_3)_2$, were used in excess. Sometimes, the solubility of the product in an excess of the inorganic precipitant was noted, *e.g.*, the solubility of ferric guanylate in FeCl_3 in which case the hydrolytically formed HCl may have participated in the reaction (4). The dissociation of nucleates in neutral salts, such as NaCl or CaCl_2 , has been occasionally reported (5). Credit for fundamental research on the solubilities of the salts of thymonucleic acid (T) with proteins and histones is due to E. Hammarsten (6). The former are water-soluble at pH 3 and at substantial concentration of T, while the latter are water-soluble if a minimum of 2 Na atoms are linked to one basic equivalent of histone and one macro mole of T. An isolated observation was made by Goto (7) who described the solubility of uric acid and purines in thymine acid, a decomposition product of thymonucleic acid (T) at acid pH.

The nucleic acids as such are free from metals (4). The traces contained in commercial products are definitely impurities, according to Levene and Bass (8) and Zittle (9).

Iron impurities in the past have been erroneously interpreted as resulting from a material consisting of cell nucleic acids and an iron compound which was considered a building block of hemoglobin. This theory was apparently supported by a communication from Ascoli (10). This investigator obtained a raw nucleic acid preparation, termed plasminic acid, by treating bottom yeast with NaOH followed by precipitation with FeCl_3 . The mother liquor contained a substance retaining iron in solution. This is a property of some metaphosphates. Ascoli clearly recognized this situation in his basic paper. His substance, which was free of nitrogen, was conclusively identified as a metaphosphate by a number of salts, especially by the pure crystallized strychnine salt. He emphasized the fact that he retained the term "plasminic acid," originally introduced by Kossel (11), without implying that his substance was a component of the nucleic acid molecule proper. Unfortunately, this fact has not been taken into account. However, the coincidental occurrence of nucleic acids and metaphosphate in yeast had already been discovered several years previously by Liebermann (12). This was conclusively proven by separation of nuclein and metaphosphoric acid, together with satisfactory analysis and typical reactions of the isolated Ba metaphosphate.¹ Kossel (11) and Ascoli (10) then confirmed the natural occurrence of metaphosphate and maintained that the metaphosphate in cells is, in one way or another, associated with the cellular nucleic acids. More recently, the same conclusion was reached by Macfarlane (13), Thannhauser, Spiegelman and others (14).

Among the *compounds of nucleic acids with organic bases*, the salts with dyestuffs, proteins, histones, and protamines were investigated. The former are of outstanding histological significance, while the latter are credited with playing a universal role in the cellular life cycle.

In the experimental part it will be shown that the unexpected characteristics of solubility observed in the inorganic nucleates have remarkable counterparts in the organic nucleates. The insoluble methylene blue nucleate is gradually and completely dissolved by sodium nucleate. The same is true of the salts of various nucleic acids with crystallized serum albumin and with protamines (see pp. 198-200).

In the course of our investigation, the problem of the ability of nucleic acids to form complex salts, or compounds of higher order, arose quite naturally. Molybdate was first tested, because of the well-known tendency of MoO_3 to form complexes with various categories of substances. Initial experiments pointed in this direction. The rotatory power of nucleates is substantially affected by molybdates. In view of the variety of phenomena mentioned above, however, the effects of a number of other substances which could not be considered as promoting complex compounds with substances of the

¹ This fundamental discovery of Liebermann is quite independent of his erroneous conclusion that nucleoproteides were nothing but protein metaphosphates.

type of nucleic acids were also investigated. This latter class of substances could not *a priori* be assumed to affect the optical characteristics of nucleic acids. Neutral inorganic salts (NaCl , Na_2SO_4), the normal salts of organic acids (mono- as well as polybasic), of sulfonic acids, amino acids, alcohols, glycols, polyhydroxy alcohols, glycerophosphate, sugars, urea, acylamides, and substances such as thiamine, pyridine, urethane, chloral hydrate, *etc.*, were tested. The optical characteristics at 23–25°C. at pH 7 as well as other pH values are affected to an unusual extent; increases and decreases of rotation, even complete inversion of the rotational direction, were observed.

The optical activity of the higher molecular nucleic acids has been observed at a relatively late date by Osborne (15) in the case of triticonucleic acid. At approximately the same time, Gamgee and Jones (16) described the unexpected dextrorotation of some nucleoproteides. With regard to an individual mononucleotide, optical activity was first observed in 1907 in the case of inosinic acid (17). Optical activity is used to trace enzymatic degradations of nucleic acids (18). Amberg and Jones (19) found that temperature and pH affected the rotatory power of Y and T.² This seems logical, as electrolytes with several ionizable groups are concerned. Feulgen (20) observed a pronounced functionality to the above factors for the optical characteristics of T. Hammarsten (6) pointed out that the decreasing rotatory values of T down to 0° under the influence of alkali and heat, as well as the partial restoration under the influence of acids, are due to phenomena of denaturation. Hammarsten did not accept Feulgen's theory that the decrease of optical activity resulted from a transition of T from a tetra- to a hexavalent state under the influence of alkali. He showed that phosphoric acid was liberated even at a pH of 6.2. Such factors of secondary changes in the nucleic acid molecule are excluded in our experiments.

A remarkable change in the rotation of Y (as Na salt) was obtained by means of *dl*-propylene glycol at 25–26°C. and at pH = 7.0 (see Table V). The nucleate was recovered from its glycol solutions by precipitation with a mixture of ethanol-dioxane. Within the limits of accuracy, this treatment caused no observable changes in the optical characteristics of the original nucleate as such.

This behavior does not represent a single instance. Pyridine, the universal solvent for osazones (21), forms compounds with the latter (22). Accordingly, the optical activity in pyridine differs substantially from the value pertaining to other solvents. However, unchanged osazones may be recovered by means of H_2O , acetic acid, toluene *etc.* (21). The same is true of the phenylhydrazone of acetylphenylcarbinol (23). The adducts of amino acids to carbohydrates, which are characterized by an optical activity other than a value corresponding to a simple mixture of the components,

² In this paper yeast nucleic acid will be abbreviated as Y, and thymonucleic acid will be represented by T.

may be readily split into the individual components (24). The same applies to hydro-tropical mixtures of an optical activity deviating from that of the components (25).

The effect of neutral salts, such as CaCl_2 , on the optical activity was first observed by Pasteur (26) in the case of *d*-tartrates, and was further investigated by Darmon (27). With regard to the sugar compounds, this phenomenon was made especially evident by Hudson as well as by Isbell and Frush (28).

Considerable changes of rotation on varying the concentration of the optically active substance and varying the solvent have frequently been observed (29) since Biot described these phenomena in 1852. According to Lcithé (30), the following should be considered in this connection: The refractive power of the substances in different solvents of varying concentration is not identical. It is a function of the polar or nonpolar nature of the solvent and of the ability of the latter type of solvents to form certain salt-like compounds.

The phenomena observed by the present authors and described on pp. 200–204, were obtained at 20–25°C. and thus are different from the phenomena observed at extremely high or low temperatures, respectively, and without any additions, in the case of gelatine (31) and sulfuric esters of mixed glycerides (32).

The polarimetric effects of apparently neutral substances added to nucleates do not differ basically from the effects of these substances on the optical activity of typical crystalloids.

Accordingly, colloidal characteristics, as regards these phenomena, do not have to be considered separately. The question assumes a different aspect with regard to the above-mentioned atypical reactions of inorganic substances. Various manuals note that the nucleic acids act as protective colloids due to their high molecular weight. This is undoubtedly correct. Studies of the original literature yielded the following facts: Feulgen (20) suspended charcoal in solutions of T (sodium salt) which he could not remove by filtration under any circumstances. The present authors succeeded in removing the charcoal by 15 min. centrifugation, by shaking with kaolin or supercel, or by addition of some dialyzed $\text{Fe}(\text{OH})_3$. Sodium thymine proves suitable for the preparation of colloidal solutions of metallic silver or palladium. (See also Takahata (33).) Thymine acid is a degradation product resulting from acid hydrolysis of T. When purines are split off, nucleosidic linkages are severed, and the resulting substance containing a free aldehyde group accordingly shows the reducing properties of a free monosaccharide. Moreover, the colloidal character of a solvent does not constitute a prerequisite for preparing inorganic colloids, as may be shown by various inorganic chemical examples (colloidal NaCl , colloidal BaSO_4 , colloidal Bredig metals, *etc.*). In view of the fact that the low molecular individual mononucleotides³ in principle show the same characteristics as the higher molecular nucleic acids (see Tables and Excerpts I–IV), other explanations are needed for the above phenomena.

The polarimetric effects seem to point in the direction of addition and complex compounds. The nucleic acids contain 4 different groups

³ The fact that the alkali guanylates are gelatinous is per se no more indicative of the colloidal nature of guanylic acid, than the existence of colloidal NaCl is for a colloidal character of HCl .

perfectly suitable for the formation of such compounds. This constitutes a wealth of possibilities which apparently have not been considered to date in this light. Whatever the value for the molecular weight of a nucleic acid may be (the statements vary between 1300 and 2,000,000 for products of various origin and preparation), a tetranucleotide consisting of 4 appropriate mononucleotides is considered the basic unit. The four mononucleotides are united by ester linkages between a sugar hydroxyl group of each mononucleotide with the phosphoric acid group of the next mononucleotide. A tetraribonucleotide of such structure, thus contains 3 amino groups, 2 pyrimidine or purine hydroxyls, 5 sugar hydroxyl groups, and 4 phosphoric acid groups with a total of 5 hydroxyl groups, totaling 15 potential complex-forming groups. The characteristic ability of amino and hydroxyl groups to form coordination compounds of whatever the resulting structural type may be assumed to be, is well established. The same is true also of the phosphoryl-hydroxyl groups.

Little attention has been given to basic phosphates (34), *e.g.*, those of calcium, beryllium, thallium, *etc.*, which belong to this type of substances. $\text{Ca}_3(\text{PO}_4)_2 \cdot \text{CaO}$ is found, not only in salamander (deposit on furnace linings) and in Thomas slag of the Bessemer converter, but also seems to occur in animal bones and—in a similar composition—in mineral phosphates such as oxy-, hydroxy-, and carbonato-apatites. A pronounced tendency to form complex compounds is encountered even in cases of inorganic phosphoric and pyrophosphoric acid (phosphoryl-phosphate) when reacting with ferric ions. Ferric iron is decolorized by H_3PO_4 , while FePO_4 and $\text{Fe}_4(\text{P}_2\text{O}_7)_3$ are soluble in Na_2HPO_4 . The same is true of $\text{Sn}_3(\text{PO}_4)_2$ and “vanadyl phosphate.” $\text{Hg}_3(\text{PO}_4)_2$ and $\text{Hg}_2\text{P}_2\text{O}_7$ dissolve in NaCl , $\text{Ca}_3(\text{PO}_4)_2$ is soluble in ammonium citrate, *etc.* In the case of an ester linkage of the phosphoric acid, this tendency is further enhanced, especially if other hydroxyl groups are present in the molecule, as in the nucleotides. Phosphotartaric (35) and phosphoglyceric acids (36) readily form complex compounds. The same is true of the phosphoric compounds of glycerol and ethylene glycol. The critical data of solubility of magnesium ribonucleate (see pp. 195–196) are paralleled by the characteristics of the alkaline earth salts of glycerophosphate and fructose diphosphate, which are more soluble at lower temperatures than at higher ones. The loss of this latter characteristic at simultaneously greatly increased solubility in the presence of an ammonium salt, which is typical of the above-mentioned salts and salts of other phosphoric esters (37,38), was also found in the Mg ribonucleate (see pp. 196). The ability to form gelatinous salts, as observed in the simple mononucleotides, is developed also to an even greater extent in both phosphoglyceric acids (38), which certainly are of low molecular weight.

Thus, remarkable phenomena are linked to the phosphoryl group, which is apparently surrounded by a field of forces. In the nucleic acids,

part of the phenomena observed are due to the effect of accumulated phosphate groups, as previously determined in the case of triphosphoric acid (diphosphoryl-phosphate) $H_5P_3O_{10}$ (39). The solubility of metal sulfides, as modified by ribo- and desoxyribonucleic acids and nucleotides (see pp. 192-194), is analogous to the similar effect induced by amino-acids and peptides, as reported recently by Neuberg and Mandl (40). The number of potentially coordinative groups in nucleic acids in a tetranucleotide and in mononucleotides is higher, and the possible combinations are more manifold, than for proteins, tetrapeptides, and aminoacids, or for any other comparable substance encountered in nature. This accounts for the variety of phenomena described, and also furnishes some indications for their biological evaluation.

EXPERIMENTAL

In all cases the nucleic acids were used in the form of their alkali salts, mostly as sodium salts and occasionally as potassium salts. Yeast nucleic acids (Y) of various origin, thymonucleic (T) as well as adenylic and guanylic acids were used.

The following symbols were used for abbreviation:

- B = sodium nucleate (yeast), C. F. Boehringer u.Söhne, Mannheim-Waldhof, Germany.
- M = sodium nucleate (yeast), Merck, Darmstadt, Germany.
- S = sodium nucleate (yeast), Schwarz Laboratories, N. Y.
- T = sodium nucleate (thymus), Dougherty Chemicals, N. Y.
- A = Na or K adenylate, Schwarz Laboratories, N. Y.
- G = Na or K guanylate, Schwarz Laboratories, N. Y.

Unless otherwise stated all nucleate solutions were made up to 5% except T which was a 2% solution.

Various yeast nucleic acids (Y) were used, since it is known (41,42) that the properties and, to a certain extent, even the composition of Y, vary somewhat with the method of preparation due to nonuniform degradation by enzymes or chemical agents. Even a fractionation of Y and T is possible (43).

A. Effect of Nucleic Acids on the Reactions of Metal Salts with Hydrogen Sulfide

To afford a better basis of comparison, all experiments were carried out with purest Na_2S instead of H_2S observing all the precautionary measures recently outlined in another paper on "An Unknown Effect of Amino Acids" (40). This procedure precludes all effects of varying pH

which may be considered practically constant, especially since a mixture of ammonium hydroxide and ammonium chloride designated as "Ammono solution"⁴ was added to all of the tests in accordance with the requirements of analytical chemistry for rapid and complete precipitation of metal sulfides. In the case of vanadium sulfide, which is soluble in Na_2S , hydrogen sulfide was generated in the mixture of components from the Na_2S with slightly less than the equivalent quantity of HCl .

Table I shows representative experiments carried out with the biometals. In the addendum, experiments with some metals of pharmacological interest are given in condensed form. The great effect of the nucleates on the solubility of the metal sulfides is obvious. In

TABLE I
Sulfides

Salt		Na_2S M/10	S	B	T	A	G	Remarks
	cc.	cc.						
CoCl_2 M/100	1	1	3					Clear for 20 hr.
	1	1		3				Clear for 20 hr.
	1	1			2			Clear for 20 hr.
	M/200	2	1			3		Clear for 1 min., then precipitate
	2	1					3	Clear for 1 hr., then precipitate
NiSO_4 M/100	1	1	3					Clear for 20 hr.
	1	1		3				Clear for 60 hr.
	1	1			2			Clear for 20 hr.
	M/200	2	1			3		Clear for 15 min., then precipitate
	2	1					3	Clear for 4 hr., then precipitate
FeSO_4 M/100	1	1	3					Clear for 20 hr.
	1	1		3				Clear for 60 hr.
	1	1			2			Clear for 20 hr.
$\text{Fe}(\text{NO}_3)_3$ M/200	2	1				3		Clear for 6 hr. (15 times diluted)
	2	1					3	Clear for 6 hr. (15 times diluted)

⁴ A mixture of 34 g. NH_3 and 53.5 g. NH_4Cl in 1000 cc. of H_2O .

TABLE I—*Continued*

Salt		Na ₂ S <i>M</i> /10	S	B	T	A	G	Remarks
	cc.	cc.						
MnCl ₂								
<i>M</i> /100	5	1	3					Clear for 20 hr.
	5	1		3				Clear for 60 hr.
	5	1			6			Clear for 15 min., then precipitate
<i>M</i> /200	2	1				3		Clear for 3 min., then precipitate
	2	1					3	Clear for 20 min., then precipitate
Zn(NO ₃) ₂								
<i>M</i> /100	5	1	3					Clear for 20 hr.
	5	1		3				Clear for 60 hr.
	5	1			2			Clear for 20 hr.
<i>M</i> /200	2	1				3		Clear for 5 min., then precipitate
	2	1					3	Clear for 7 min., then precipitate.
CdCl ₂								
<i>M</i> /10	0.4	2			10			Clear for 72 hr., colorless solution
CuSO ₄								
<i>M</i> /100	1	1	3					Clear for 20 hr.
	1	1		3				Clear for 60 hr.
	1	1			2			Clear for 20 hr.
	1	1				3		Clear for 20 min., then precipitate
	1	1					3	Clear for 1 hr., gradually precipitate afterwards

ADDENDUM

All controls were brought to the same volume as the tests by addition of water. They yielded immediate precipitates of the various metal sulfides. Four drops of "ammono solution" were added to all of the tests with the exception of the cadmium tests.

Similar tests, with analogous results, were carried out in various concentrations with H·COOTI, In₂(SO₄)₂, AgNO₃, HgCl₂, (CH₃·CO₂)₂Pb, SnCl₄, UO₂(NO₃)₂, and NH₄VO₃. In the vanadate tests slightly less than the equivalent quantity of *M*/10 HCl, as referred to the applied Na₂S solution, was added. Whereas the yeast nucleates yielded a clear brown solution with thallium formate, thymonucleate formed a ruby red clear solution of intense color.

many cases, the precipitation of the metal sulfides was completely prevented, while, in other cases, the precipitation was delayed for protracted periods of time. If these reactions are to be interpreted as complex formation, it might be assumed that these complexes are stable

for many hours or, due possibly to intramolecular rearrangement, for more limited periods of time. In the examples described, the solubility of the metal sulfides in the nucleates is of such magnitude that it should not be neglected in the consideration of biochemical processes. The same is true for mercaptides. Mercaptans, as well as H_2S , are frequently encountered products of metabolism.

*B. Effect of Nucleic Acids on the Precipitation of Metals by
Phosphate, Arsenate, Carbonate, Silicate, Fluoride,
Sulfate, Borate, Chromate, etc.*

A number of experiments have been condensed from tables to give the *Excerpts* II–IV. It is shown that the normal precipitation reactions

EXCERPT II

The alkaline earths were tested with regard to prevention or delay of their usual precipitation reactions: Beryllium phosphate and carbonate were kept in solution for many hours by the nucleates S, B and T.

Magnesium ammonium phosphate and MgCO_3 remained in solution for from 20 min. to 4 hr. with S and T. B was somewhat less effective with regard to prevention of the precipitations.

Calcium phosphate was solubilized by S for 20 hr., and by A for 6 hr., while S, B and T, as well as A and G, kept calcium fluoride in solution for 24 to 60 hr. The 3 nucleates also yielded CaCO_3 solutions which remained clear for 6 to 60 hr.

Similar results were obtained for strontium and barium. All of the nucleates, as well as A and G, solubilized even barium sulfate for 5 to 24 hr. In the presence of the mentioned nucleate solutions precipitation of BaSO_4 from 1 cc. each of $M/100$ BaCl_2 and $M/100$ Na_2SO_4 is prevented. 10 cc. are required in the case of B, M, S and T, 20 cc. in the case of A and G. The nucleate solutions are added to the Na_2SO_4 before precipitation with BaCl_2 . In the nucleate-free control experiments glittering BaSO_4 precipitates at once.

EXCERPT III

Phosphate, arsenate, borate and carbonate reactions were carried out on a group of metal salt solutions comprising Mn, Zn, Cd, Al, Fe^{++} , Fe^{+++} , Co, La, Ce, and In. S, B and T, as well as A and G, were used in these tests. The typical precipitation reactions were again significantly delayed or prevented. The two yeast nucleates generally yielded similar results. In the case of cadmium phosphate, no prevention of precipitation was obtained by the yeast nucleates, whereas the solution remained clear for 72 hr. with T. A and G also proved effective in preventing precipitation in various instances; the effective periods, however, were less protracted than those obtained with S, B and T.

EXCERPT IV

Many of the typical heavy metal precipitation reactions are affected by the presence of nucleates:

Cu: The precipitation of copper phosphate or carbonate is prevented for periods of 24 hr. or more in the presence of all nucleates and nucleotides.

Bi: Solutions of $\text{Bi}(\text{ClO}_4)_3$ remained perfectly clear on addition of KI solution for 5-30 min. in the presence of A and G, and for 60 hr. in the presence of S and B.

Pb: Phosphate, carbonate, sulfate, and borate precipitations are prevented by the presence of yeast nucleates for 5-20 hr.

Hg: All nucleates, as well as adenylate and guanylate, prevent the formation of the glittering crystalline red precipitate (mercuric oxychloride), ordinarily obtained by adding Na_2CO_3 solution to HgCl_2 . Tests remain clear for 5-20 hr.

Ag: The typical reactions with phosphate, arsenate, carbonate, and borate are prevented by all types of nucleates and nucleotides for periods of 5-24 hr.

Ti: TiCl_4 (*M*/10 solutions in 30% ethanol) remain clear on addition of Na_2CO_3 or Na_2HPO_4 for several hours in the presence of yeast nucleates.

Cr: The characteristic precipitation produced by BaCl_2 in K_2CrO_4 was delayed for 20 min. by yeast nucleates.

Mo: The precipitation of calcium or barium molybdate did not take place in the presence of any of the nucleates or nucleotides for periods of 5-8 hr. In some instances the tests remained clear, even when heated for 60 sec. on a boiling water bath.

are prevented or altered. Of the numerous experiments made, only those which are concerned with bioelements or substances of pharmacological interest are mentioned. The solubility of calcium phosphate, calcium carbonate, calcium fluoride, magnesium ammonium phosphate, barium sulfate, barium molybdate, and the corresponding compounds of other alkaline earths in nucleates, seems impressive. The solubilization of the phosphates and carbonates, and the abnormal solubility of some basic salts as regards the biometals and metals of pronounced pharmacological activity (Mn, Zn, Fe, Co, Ni, Cu, Ag, and Hg), should be emphasized. It should furthermore be pointed out that the almost insoluble compounds of Be, La, In, Ce^{III} , and Ti^{IV} with nucleic acids are distinctly soluble in alkali nucleates. Such solutions are either no longer precipitable by alkali carbonates or phosphates, or else precipitate only after many hours.

FURTHER REMARKS

Magnesium Compounds. Pyridine

Five cc. of 5% solutions of S or B with 15 cc. of 25% *Mg acetate* solution remain clear at room temperature, but become cloudy when heated beyond 37°C. Such cloudiness increases over a range of 40-65°C. and then decreases, disappearing at around

80°C. On cooling, cloudiness reappears around 70°C. and clears up completely below 37°C. NH_4Cl , when added to the mixture of Mg acetate and nucleate, does not affect the phenomenon, while NH_4 acetate prevents it. The alternating effects of cloudiness on heating and clearing on cooling may be repeated several times. However, continued heating in a boiling water bath for 6 min. destroys the above phenomenon, and the nucleates no longer show the cloudiness dependent on temperature.

With 25% MgCl_2 solution the phenomena observed for S or B are similar to those obtained with Mg acetate. A difference exists inasmuch as a 20 min. heating period in a boiling water bath does not completely eliminate the critical temperature limits for the formation of a precipitate.

The above is in no way related to the observation by Hammarsten (6) that free T is precipitated at room temperature by less than 0.02 equivalent of MgCl_2 .

Five cc. of 5% S with 15 cc. of *pyridine* show a phenomenon similar to that of Mg acetate. The mixture becomes cloudy on heating and clarifies again after cooling. A 15 min. heating period in a boiling water bath destroys the phenomenon and a separation into 2 layers is obtained. It appears that pyridine is salted out by the products presumably formed by hydrolysis of the nucleate in a Bredereck degradation (43), especially sodium phosphate.

Molybdate

1 cc. $M/10 \text{ Na}_2\text{MoO}_4$, 5 cc. 5% S or M or 2½% K adenylate, 1 cc. $M/10 \text{ Ca}(\text{NO}_3)_2$; heated on a water bath for one minute to approximately 50°C. All three tests clear. Control with 5 cc. H_2O instead of nucleate: white precipitate. Approximately 0.02 g. *ammonium phosphomolybdate* dissolved at room temperature in just sufficient $N \text{ NaOH}$. Then $N \text{ H}_2\text{SO}_4$ added until acid reaction. Solution remains clear at room temperature, while yellow precipitate reappears after 15 min. heating on the water bath. This reappearance of precipitate may be prevented by adding 5 cc. B to clear cold acid solution and subsequent heating on water bath. The precipitate obtained in the above control experiment by heating on water bath without addition of B may be redissolved by heating it with B on water bath.

Solubilities of Some Metal Nucleates

The precipitate obtained from 1 cc. 5% S with 20 cc. $M/100 \text{ CuSO}_4$ was centrifuged and washed with distilled H_2O : Green precipitate was soluble in 2 cc. 5% S. Green precipitate was insoluble in 2 cc. $M/100 \text{ Na}_2\text{SO}_4$. Analogously obtained precipitate from CuCl_2 proved soluble in 2 cc. 5% S and partly soluble in 6 cc. $M/10 \text{ NaCl}$.

Precipitate obtained from 5 cc. $M/10 \text{ Fe}(\text{NO}_3)_3$ + 1 cc. 5% S:
Pink precipitate soluble in 3 cc. 5% Y

Pink precipitate insoluble in 5 cc. $M/10 \text{ NaNO}_3$

Pink precipitate insoluble in 5 cc. $M/10 \text{ NaCl}$

6cc. $M/10$ $CdSO_4$ or $CdCl_2$ with 1 cc. 5% S:

White precipitate soluble in 3 cc. 5% S

White precipitate soluble in 3 cc. $M/10$ NaCl

White precipitate soluble in 5 cc. $M/10$ Na_2SO_4

4 cc. $M/100$ $Ce(NO_3)_3$ with 1 cc. 5% S:

White precipitate soluble in 3 cc. 5% S

White precipitate insoluble in 5 cc. $M/10$ $NaNO_3$

White precipitate insoluble in 5 cc. $M/10$ NaCl

Ceric sulfate forms a precipitate with S which proved practically insoluble in excess nucleate.

Stannic chloride also forms a heavy precipitate, which however was more soluble in S.

6 cc. $M/100$ $Cr_2(SO_4)_3$ with 1 cc. 5% S:

White precipitate soluble in 2 cc. 5% S (disregarding traces of residue)

White precipitate insoluble in 4 cc. $M/100$ Na_2SO_4

6cc. $M/10$ $UO_2(NO_3)_2$ with 1 cc. 5% S:

Yellow precipitate soluble in 3 cc. 5% S

Yellow precipitate insoluble in 3 cc. $M/10$ $NaNO_3$

If half-saturated solution of K_2CO_3 is added to $M/10$ uranyl nitrate, the precipitate originally formed is dissolved. The addition of 5% S or B to this solution does not cause precipitation. K_2CO_3 also dissolves a preformed precipitate of uranyl nucleate.

Maximum solubility of *ferric phosphate* in S and B:

5 cc. 5% S

5 cc. 5% B

6 cc. $M/100$ $Fe(NO_3)_3$

6 cc. $M/100$ $Fe(NO_3)_3$

3 cc. $M/50$ Na_2HPO_4

3 cc. $M/50$ Na_2HPO_4

Clear for about 20 min.

Further additions of Fe^{+++} cause immediate cloudiness in the above tests.

Ferrous sulfate proved unsuitable for these tests since only minor amounts of the phosphate could be kept in solution.

Maximum solubility of $CaHPO_4$ in S: 5 cc. 5% S, 5 cc. $M/100$ $CaCl_2$, 1 cc. $M/10$ Na_2HPO_4 ; clear for 25 min., subsequently slightly cloudy. Further addition of $CaCl_2$ results in immediate cloudiness.

Vanadyl Sulfate

White precipitates soluble and colorless in excess nucleates are obtained from 1% solution of vanadyl sulfate with 5% solutions of S or B or sodium guanylate or with 2% T. Neither Na_2CO_3 nor Na_2HPO_4 precipitates these solutions. The alkaline carbonate solutions turn yellowish-green and subsequently olive to violet on storage at room temperature. The control precipitate obtained by Na_2CO_3 with pure $VOSO_4$ is insoluble in an excess of precipitant. Na_2HPO_4 precipitates $VOSO_4$. The resulting precipitate, remarkably, is readily soluble in excess disodium phosphate at $45^\circ C.$, and somewhat more slowly at room temperature.

Metavanadate

- (1) 0.2 cc. $M/10$ $BaCl_2$
 2.0 cc. 5% sodium guanylate
 2.0 cc. $M/100$ NH_4VO_3
- (2) 0.2 cc. $M/10$ $BaCl_2$
 2.0 cc. 5% T
 2.0 cc. $M/100$ NH_4VO_3
- (3) 0.2 cc. $M/10$ $BaCl_2$
 2.0 cc. 5% S
 2.0 cc. $M/100$ NH_4VO_3
- (4) 0.2 cc. $M/10$ $BaCl_2$
 2.0 cc. 5% B
 2.0 cc. $M/100$ NH_4VO_3
- (5) 0.2 cc. $M/10$ $BaCl_2$
 2.0 cc. H_2O (Control)
 2.0 cc. $M/100$ NH_4VO_3

(1) to (4) remain completely clear for 24 hr., even after heating; (5), the control, shows a flaky white precipitate which likewise cannot be dissolved by heating.

The *borates* of Hg and Ag, known to be nearly insoluble, are easily dissolved in both B and S, in the case of Hg after slight warming. The same is true for ferric *silicate*. The precipitation of Ca and Mg silicate is prevented by the nucleates.

Practically the same results were obtained when the alkali nucleates were replaced by soluble *salts of the nucleic acids with organic bases*. This can be shown for the neutral salts with methylamine, ethanolamine (colamine) and many others. The nucleate of hexamethylenetetramine, which does not occur in nature, also shows similar solubilizing effects.

In this connection it may be mentioned that nucleates of alkalis and of organic bases prevent the precipitation of *alkaline earth soaps* and redissolve them once formed. This fact merits attention in view of the various processes in which fatty acids take a part.

Solution of one compound in a nucleate does not prevent solubilization of another. For instance, $CaCO_3$ or $CaHPO_4$ can still be dissolved in a "solution" of $BaSO_4$ in B.

C. Effect of Alkali Nucleates on Dyestuff Nucleates

Tests:	Controls:
(1) 0.5 cc. 1.7% S	0.5 cc. 1.7% S
5 cc. 0.1% methylene blue	5 cc. 0.1% methylene blue
5 cc. 5% S	5 cc. H_2O
clear.	precipitate.
(2) 0.5 cc. 1.7% M	0.5 cc. 1.7% M
5 cc. 0.1% methylene blue	5 cc. 0.1% methylene blue
5 cc. 5% M	5 cc. H_2O
clear.	precipitate.

(3) 0.5 cc. 2% T	0.5 cc. 2% T
5 cc. 0.1% methylene blue	5 cc. 0.1% methylene blue
5 cc. 2% T	5 cc. H ₂ O
clear.	precipitate.

In a recent excellent interpretation by L. Michaelis (44) of the interaction of basic dyestuffs with nucleic acids, no mention was made of this phenomenon, nor were indications thereof found in the older literature on the compounds of basic dyestuffs with nucleic acids. The various authors (20, 45) added the nucleate solution to an excess of dyestuff solution and thus could not observe the solubilizing effect of the former. It is quite possible that alkali nucleates are present in the cells and it would then be conceivable that they affect the aspects of microscopic staining technique, vital staining, and cytochemical demonstration methods.

*D. Effect of Alkali Nucleates on the Precipitation of
Protamine and Albumin Nucleate⁵*

Protamine sulfate:

Tests:	Controls:
(1) 3 cc. 5% S	0.5 cc. 1.7% S
8 cc. 0.11% protamine sulfate	2.5 cc. H ₂ O
clear.	8 cc. 0.11% protamine sulfate
	heavy precipitate.
(2) 4 cc. 5% M	0.5 cc. 1.7% M
8 cc. 0.11% protamine sulfate	3.5 cc. H ₂ O
clear.	8 cc. 0.11% protamine sulfate
	heavy precipitate.
(3) 5.25 cc. 2% T	0.25 cc. 2% T
5 cc. 0.11% protamine sulfate	5 cc. H ₂ O
clear.	5 cc. 0.11% protamine sulfate
	heavy precipitate.

Plasma albumin (Cryst. Bovine Plasma Albumin Armour):

Test:
(1) 3 cc. 5% M
0.5 cc. 5% plasma albumin
0.5 cc. 5% M (free acid)
2 drops 10% acetic acid
clear.

⁵ We have observed a similar behavior in the case of streptomycin nucleates described by S. S. Cohen, *J. Biol. Chem.* **166**, 393 (1946); **168**, 511 (1947).

Control:

3 cc. H_2O

0.5 cc. 5% plasma albumin + 1 drop 10% acetic acid

0.5 cc. 5% M (free acid) + 1 drop 10% acetic acid

heavy precipitate (soluble in 5 cc. 5% M).

Test:

(2) 6 cc. 2% T

0.5 cc. 5% plasma albumin

0.5 cc. 2% T (free acid)

2 drops 10% acetic acid

clear.

Control:

6 cc. H_2O

0.5 cc. 5% plasma albumin + 1 drop 10% acetic acid

0.5 cc. 2% T (free acid) + 1 drop 10% acetic acid

heavy precipitate.

In this connection an unrelated remarkable observation of Greenstein (46) should be mentioned: Investigating the effect of T on the heat-stability of proteins, the author found that T—in contradistinction to Y—prevents the heat coagulation of egg albumin. In this case T exerts an effect similar to a hydrotropic substance, which likewise prevents the coagulation of proteins (47). The comparison with hydrotropic substances actually is not as far-fetched as it may seem, in view of the fact that phosphoric esters of simple structure frequently proved especially active hydrotropic materials of structural specificity (48). The action of ATP on muscle proteins should also be mentioned. According to Szent-Györgyi (49), ATP increases hydration and solubility of myosin. In the case of actomyosin, a compound of the proteins actin and myosin in not exactly determined proportions, it causes dissociation of the two components and increased sedimentation of a granular precipitate.

E. Phenomena of Combination of Inorganic and Organic Substances with Nucleates as Manifested by Polarimetric Effects

Tables V and VI record observations on the partly significant changes in optical activity of the alkali nucleates on addition of neutral salts, salts of various organic acids and bases, various homologous glycols, amino acids, mixtures of amino acids, acyl amides, sugars, aliphatic and aromatic sulfonic acids, pyridine, urethane, chloral hydrate, furfuryl alcohol, *etc.* The change of rotation is greatest on addition of ethylene glycol and its derivatives and homologues. Since 5–10% solutions of the sodium nucleates are miscible with the water-free glycols, the series ethylene glycol, *dl*-propylene glycol and *meso*-butylene glycol could be tested. The maximum effect was obtained by means of *dl*-propylene glycol, a decrease of rotation from $+10.3^\circ$ to -2.6° having been observed in this case. (Water-free 1,3-butylene

glycol, methyl-1,3-pentanediol, glycerol- α , β -dimethyl ether and diacetone alcohol could not be mixed with the nucleates, as precipitates or semi-solid materials are formed.) Trimethylene glycol causes a change of rotation from positive to weakly negative; *dl*-glycerol- α -methyl ether changes the dextrorotation to a strong levorotation somewhat like *dl*-propylene glycol, which is structurally similar. Hardly any effect could be observed with guanidine hydrochloride; a decrease in rotation of 9% took place with nicotine, and sodium *n*-butyl sulfonate showed a decrease of 28%. Thiamine HCl reversed the dextrorotation of B to yield a weakly levorotatory solution. In the case of S the experiment can be completed only after preliminary heating of the solution. The results obtained are analogous. A definite decrease in rotation is also obtained with sodium guanylate. This formation of an addition compound of a vitamin and nucleate appears worthy of note. Obviously, increases, as well as decreases, in optical activity were obtained with the various classes of substances.

For polarization determinations, the commercial Y preparations should be suitably purified by repeated precipitations of the sodium salts. Five per cent aqueous solutions of Y are precipitated by twice their volume of dioxane. Perfectly filtrable solid flakes are thus obtained. These are filtered by suction and washed with ethanol and ether. If acetone is used as a precipitant, cloudy solutions will frequently result. A flaky precipitate, however, may be obtained from these cloudy solutions by the addition of a drop of methanol solutions of LiCl, BaBr₂, or Ba(ClO₄)₂.

For all determinations of rotation a saccharimeter with Ventzke scale and 2 dm. tubes were used. (The conversion factor for angular rotation amounts to 0.346.) In all cases, 15 cc. of the inorganic or organic solutions were added to 5 cc. of the nucleate; in the controls 15 cc. of H₂O were added instead.

The number and variety of substances which, according to the polarimetric tests, enter into reaction with the nucleates was surprisingly large. This seems indicative of unusual coordinative or associative forces of the nucleates which, as such, represent exceedingly complex substances.

F. General Remarks and Discussion

The special role of nucleotides in enzymatic systems has been thoroughly explored. In 1924, E. Hammarsten (6) defined the significance of nucleic acids for protoplasm and for cell nuclei. In classical investigations on thymonucleic acid this author showed how the physico-

TABLE V
Comparative Polarimetric Values of Nucleate complexes^a

5 cc. 5% S	15 cc. H ₂ O	50% LiBr	M NaCl	M Na ₂ SO ₄	2M K ₂ CO ₃	50% KJ	M KSCN	50% NH ₄ SCN	M K ₃ HPO ₄
5% S	+6.0° pH 7 ^b		+10.5°	+10.5°		+9.3°	+9.3°		
5% B	+5.1°	+6°	+7.9°	+8.2°	+1.1°		+7.7°	+3.3°	+6.8°
5% S	+6.0°	M/2 Na pyro- phosphate +6.9°	M Na ₂ MoO ₄ +7.8°	M K ₂ B ₄ O ₇ +5.9°	50% Na acetate +10.8°	66% Na diac- etate +10.1°	Na citrate +10.1°	25% Mg acetate +9.9°	{ 5 cc. H ₂ O pH 15 cc. Mg acetate = 7.4
5% B	+5.1°	+4.8°	+8.4°	+5.8°			+7.5°	+7.5° pH 7.5	
2% T	+1.0° pH 8		+3.7°	Oily separation			+8.0°		
5% S	+6.0°	M Na benzoate +10.9°	M Na salicylate +8.9°	40% Na o-xylenesulfonate +8.9°					
5% B	+5.1°	+6.8°	+6.2°						

^a The designation "complexes" was used without implication as to the actual chemical structure.

^b With the exception of the cases especially marked in the tables, the pH was adjusted to about 7 by suitable neutralization. The natural alkalinity was maintained in the experiments with potassium and guanidinium carbonates.

TABLE V—Continued

5% S	+6.0°	M Ethylene glycol +5.8°	Pure ethylene glycol	M Propylene glycol +5.3°	Pure propylene glycol	M 2,3-meso-butylene glycol +5.1°	Pure 2,3-meso-butylene glycol	66% Methylcellosolve
5% B	+5.1°	+3.4°	-1.3°	+4.7°	-1.7°	+4.6°	+1.05°	
10% B	+10.3°		-1.9°		-2.6°			+2.0°
5% S	+6.0°	3M Glycerol	Pure glycerol	M Na di-glycero-phosphate				
5% B	+5.1°		±0°	+6.4°				
10% B	+10.3°	+3.1°						
5% S	+6.0°	6% Glucose +18.7° 15 cc. Glucose 5 cc. H ₂ O +13.5°		10% Mannitol +5.4° 15 cc. Mannitol 5 cc. H ₂ O ±0°	50% Sorbitol +6.0° 15 cc. Sorbitol 5 cc. H ₂ O +1°	66% Furfuryl alcohol	5M Chloral hydrate	
5% B	+5.1°					+2.0°	-1.2°	
5% B	+5.1°	2M Urea +3.5°		6M Urethane +2.0°	10M Formamide +1.8°	6M Acetamide +1.8°	3.5M Glycocoll +4.4°	
5% S	+6.0°	20% Na glutamate +4.4° 15 cc. Na glut. 5 cc. H ₂ O = -2.7°	50% Na glutamate +10.7° 15 cc. Na glut. 5 cc. H ₂ O = -0.2°	L-Arginine HCl (5 g. in 15 cc. H ₂ O) +20.0° 15 cc. argin. 5 cc. H ₂ O = +1.4°	Acid hydrolyzate casein -4.3° 15 cc. ac. hydr. 5 cc. H ₂ O = -13.4°	Tryptic hydrolyzate fibrin -15.5° 15 cc. tr. hydr. 5 cc. H ₂ O = -23.2°	50% Cholin HCl +5.4°	50% Pyridine +1.3° pH 7.3
5% B	+5.1°			+18.0°			+3.5°	+0.3°

TABLE VI
Comparative Polarimetric Values of Nucleotide Complexes

5 cc.	15 cc. H ₂ O	15 cc. M NaCl	15 cc. M K ₂ B ₄ O ₇	15 cc. M Na ₂ MoO ₄	15 cc. Pure propylene glycol
5% K guanylate	-2.8° pH 7.5				±0° pH 7.0
5% K adenylate	-4.0° pH 6.9				-7° pH 6.5
5% Na guanylate	-3.4° pH 9	-3.6°	-2.7° pH 8.2	-3.0° pH 8.2	-3.8° pH 9
	-4.2° pH 8.2				
5% Na adenylate	-4.8° pH 7	-4.8°	-4.5° pH 7.5	-4.4° pH 7.5	-6.2° pH 7
	-4.6° pH 7.5				

chemical qualities of this substance maintain the requisite heterogeneity for cellular life. Subsequently, the role of nucleic acids in numerous biological problems has been investigated, *e.g.*, in problems of immunology and infection, genetics, mutation and transformation of microbes, chromosome division, mitosis and meiosis, autotynthesis and self-multiplication of genes, virus reproduction, embryonic development and metabolism, cell regeneration and tissue growth, reproduction of normal and neoplastic tissues, the mechanism of protein and enzyme synthesis, inhibition of PGA conjugase activity, treatment of infectious diseases, and of radiation therapy of any type. The latter are a continuation of previous work on changes in the nucleate molecule by sunlight (50) and should establish experimental modifications of nucleic acid systems in the cell, especially in tumor cells, and thus in the cellular organization, *etc.* Many biological effects may be attributed to an enormous number of isomers which may result from internucleotide linkages (polymerization or polycondensation of tetranucleotide units into polynucleotides). According to X-ray studies of Astbury (51), however,

there is a certain limitation in the number of the potential resulting substances if the macro molecule is characterized by an unbranched chain of oligonucleotides only, and if branched chains are absent. On the basis of the experiments described in the present paper, an almost infinite number of variations of a different type may be conceived: The nucleic acids may react with a large variety of inorganic or organic substances. Even the unit of a ribo-tetranucleotide is characterized by 15 reactive groups. Such an accumulation of reactive and heterogeneous groups facilitates an enormous variety of combinations according to the potential selection of type or number. The polyvalent nucleic acids may be compared somehow to the heteropolyacids (A. Rosenheim), or the compounds produced by intramolecular forces may be considered as complex, chelate or molecular compounds, as adducts, coordination or adsorption compounds, or as substances linked by covalence forces. The transitions are fluid, and definite border lines may not be drawn at this time. However, the universal effects certainly lend considerable significance to these processes. The number of reactive and multifunctional substances suitable for tests is almost unlimited. Analogous effects may be obtained by variation of the quantities of the components in the individual reactions. The experiments recorded here only represent selected examples of the protocols for the simplest test setups.

Under the dynamic conditions existing in the living cell, effects may occur at minimum concentrations which, in significance, may exceed those obtained in the experiments under static conditions.

The variety of possibilities is furthermore enhanced by the fact that many cells contain both types of nucleic acids. This is true not only of animal tissues (see Schmidt and Thannhauser, 52), including chromosomes (Mirsky, Pollister and Ris, 53), but also of higher and lower plants (Feulgen and Rossenbeck, 54) as found at an early date, and of bacilli (Bendix, Boivin and Vendrely, Coghill and Johnson, Ruppel, Sevag, Stacey *et al.*, 55). The existence of mixed ribo- and desoxyribonucleic acids has also been discussed (56).

Assuming that each of the 15 addenda is present in sufficient amount to react with any or all of the functional groups, and considering the further possibility that none of the 15 groups takes part in the reaction, the number* of possible combinations for the ribose tetranucleotide unit turns out to be of the order of 16^{15} ; this equals 10^{18} . Since, however,

* We appreciate the help of Mr. D. Smith and Mr. F. P. Callahan, both of Interchemical Corp., N. Y., in checking the numerical problem.

more than 15 addenda (A) are conceivable, the number of possible compounds (A^{15}) increases to the immeasurable. In view of this fact, the symmetry of certain OH groups in the formula preferred by Tipson (1) and the possible diminution of isomers resulting from this symmetry plays a minor part. The number of isomers increases if hydrogen bonding and van der Waals' forces on the long chains are taken into account, since they produce new states of the molecule, while association of the addenda would create centers for increased coordination, quite apart from the fact that each addendum might be polyvalent as in bases, acids, glycols, *etc.* Hypotheses on possible changes in the constitution of the chain molecule and alteration in the order of the components within the tetranucleotide unit may be omitted from consideration. Obviously, the addenda could be proteins, but it is not absolutely necessary to refer to nucleoproteins for an explanation of the manifold biological effects of nucleates. Even without participation of proteins the unlimited variability appears assured by the results reported and can be extended experimentally to any desired degree.

Furthermore, it is obvious that a "deionization" of mineral substances is highly significant for metabolism. Thus, the biometals are protected from precipitation as sulfides, the same metals are solubilized in the form of phosphates, borates, silicates and carbonates, and calcium carbonate, calcium phosphate and calcium fluoride, as well as other compounds of the alkaline earths and other metals, are dissolved by the nucleates as shown in the tables and excerpts. It seems noteworthy that these phenomena might facilitate the transport of insoluble or nearly insoluble substances throughout the organisms. For cellular metabolism it seems just as significant that the carrier and transport functions of the nucleates are destroyed by enzymatic degradation of the latter with the effect that the solubilized substances may be unloaded at other points. A continuous and reversible exchange of loosely-bound addition complexes of nucleates is also possible. With regard to cellular poisons of anionic or cationic character, narcotics, alkaloids and related substances, vitamins, antibiotics, and compounds of special pharmacological activity, combinations with nucleic acids were proved to exist by various methods.

It seems conceivable that the combination of nucleic acid with any type of substance may result in permanent or, significantly, temporary specificities capable of promoting a variety of reactions to a greater extent than those known for enzyme systems (apoenzyme, coenzyme +

inorganic or organic activator). It seems logical that the formation of nucleate complexes may modify the rate of diffusion of permeating substances and may regulate competitive actions according to intensity and persistence of linkage. For all these effects it is irrelevant whether aggregation of nucleotides or depolymerization of macro molecules is involved.

Apart from the activity in the cell organism, the nucleic acids may decisively affect other natural processes: The transport of calcium carbonate and phosphate, barium sulfate, iron, manganese, magnesium, silicon, boron, vanadium, and molybdenum⁶ compounds in the soil should be considered here. Petit (59) found nucleic acid in the humus at an early date. This was verified by Schreiner (60) as well as by Koch and Oelsner (61). From the work of the last two authors, it follows that nucleic acids of animal origin, and especially those derived from plants, are conserved intact in the soil for many months, being decomposed by the normal soil bacteria only by a slow process. The role of the nucleic acids in the formation of certain metallic compounds extractable from the soil by suitable solvents should be experimentally investigated. The same applies to problems of nitrification in soil where biometals are involved.

In summary, it is evident that the nucleotides and nucleic acids might participate in numerous processes of vital interest to an extent, and in a way, not heretofore considered.

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CONCLUSIONS

In the presence of nucleic acids and nucleotides many inorganic or organic substances of some biochemical significance are subject to

⁶ The molybdenum content of the soil has proved of special interest lately. The earliest references by Demarçay and von Oefele (57), and the most recent reference on the biochemical significance of this element by Elvehjem and associates (58), should be mentioned in this connection.

modification of their properties and show unusual behavior. Reciprocally, an infinitely large number and variety of reactions in the living cell and in the soil may result thereby.

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Studies on the Respiration of the White Potato.¹
III. Changes in the Terminal Oxidase Pattern of
Potato Tissue Associated with Time
of Suspension in Water

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INTRODUCTION

It has previously been shown (1) that the respiration of potato tissue (*Solanum tuberosum* L.) involves cytochrome oxidase. Another terminal oxidase, exhibiting marked sensitivity to lowered partial pressures of oxygen but little or no reaction to carbon monoxide, is probably also involved. Both enzymes have been demonstrated in homogenates prepared from the washed potato slices employed in the respiration studies.

Early in these investigations it had been noted that the sensitivity of potato respiration to CO and to lowered pO_2 was dependent upon the length of time that the tissue was washed prior to use in the respiratory experiments.² To elucidate the nature of this dependency, we undertook a study of the relation of the length of the washing period to the respiration of the potato slices as well as to the activities of their homogenates with respect to the above-mentioned oxidases. The results of the study are given in this paper.

¹ The research which this paper reports was undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views of the War Department.

² As a result of this observation, a standard washing time of 18–22 hr. was used to obtain the results reported in the previous paper (1).

EXPERIMENTAL

The materials and methods employed in this work are the same as those given in the previous paper (1), except where noted.

To determine the respiration of the intact potato slice, the oxidase activity of the homogenate prepared from it, and the possible effect of prolonged washing on both, 4 similar experiments were performed as follows. A large number of slices, 7 mm. in diameter and 0.5 mm. thick, were prepared and put to wash in running tap water at 14°C. Aliquots of the slices were removed after a few min. washing, after 18–20 hr. of washing, and after 94 hr. of washing. Each aliquot was then divided into two parts. One part was used for the determination of the rate of respiration of the slices in gas mixtures of 20% O₂–80% N₂ and of 2.5% O₂–97.5% N₂. The results of one experiment are shown in Fig. 1. The other part was used for the preparation of homogenates.

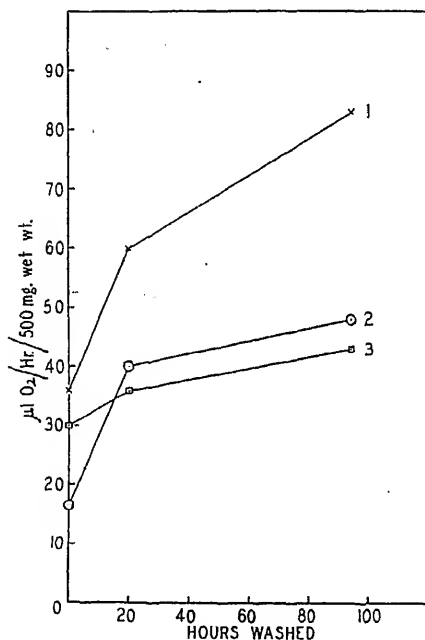


FIG. 1. Variation in respiration with length of washing period. Each point represents an average value obtained from duplicate vessels containing 25 slices (500 mg. wet weight) in 2 ml. *M*/100 phosphate buffer, pH 7.4. The midwells contained 0.2 ml. of 20% KOH. Curve 1. Respiration in 20% O₂–80% N₂. Curve 2. Per cent inhibition of respiration by lowered pO₂ (2.5% O₂ vs. 20% O₂). Curve 3. Respiration in 2.5% O₂–97.5% N₂.

With *p*-phenylenediamine (ppda) as the substrate, the cytochrome oxidase activity, as well as the oxidizing activity of the homogenates without added cytochrome, were determined in 20% O₂-80% N₂ and in 2.5% O₂-97.5% N₂. *p*-Cresolase activity was determined in 20% O₂-80% N₂. Fig. 2 shows the results of these enzyme assays. While the data represented in Figs. 1 and 2 were obtained from the same experiment, the other 3 experiments gave very similar results.

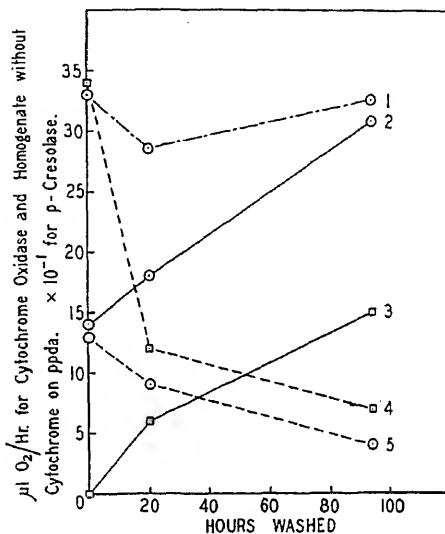


FIG. 2. Variation in enzyme activity with length of washing period. The homogenate action on ppda was determined in vessels having 1 ml. of homogenate (200 slices/6 ml. H₂O), 1 ml. *M*/10 phosphate buffer, pH 7.4, 0.4 ml. H₂O in main rooms; 5 mg. ppda in 0.5 ml. H₂O, pH 6.0, in side arm; 0.2 ml. 20% KOH in midwell. Cytochrome oxidase figures represent difference in O₂ uptake between these vessels and vessels in which 0.4 ml. 2×10^{-4} *M* cytochrome *c* was used in place of the 0.4 ml. of H₂O. *p*-Cresolase activity was determined on 1 ml. homogenate, 1 ml. *M*/10 acetate buffer, pH 5.5 in main room; 4 mg. *p*-cresol in water in side arm; 0.2 ml. 20% KOH in midwell. Curve 1. *p*-Cresolase. Curve 2. Homogenate on ppda, (20% O₂). Curve 3. Homogenate on ppda, (2.5% O₂). Curve 4. Cytochrome oxidase, (2.5% O₂). Curve 5. Cytochrome oxidase, (20% O₂).

It will be seen from an examination of Figs. 1 and 2 that the action of the homogenate on *p*-phenylenediamine increases 120% over the 94 hr. of washing, and that, at the end of the 94 hr., this action is diminished 53% by lowering the pO₂ from 0.2 to 0.025 atm. During this time the respiration of the slices rises 130%, while the decrease in respiration

consequent to a reduction in pO_2 from 0.2 to 0.025 atm. reaches 48% after 94 hr. Over this period, the cytochrome oxidase activity of the homogenate decreases. The cytochrome oxidase activity is certainly not inhibited by the lower partial pressure of oxygen, but, on the contrary, appears to be somewhat elevated. Homogenate action on *p*-cresol shows no upward or downward trend.

A possible explanation for the phenomena represented by the data of Figs. 1 and 2 is the following. There are 2 enzymes capable of functioning as terminal oxidases in the potato tuber. The activity of one, cytochrome oxidase, is not diminished by lowered pO_2 , while the activity of the other is very sensitive to lowered pO_2 . For convenience, this latter enzyme shall be referred to as enzyme X. With increased periods of washing, the activity of cytochrome oxidase per unit of potato tissue decreases, as does its relative participation in the respiration of the

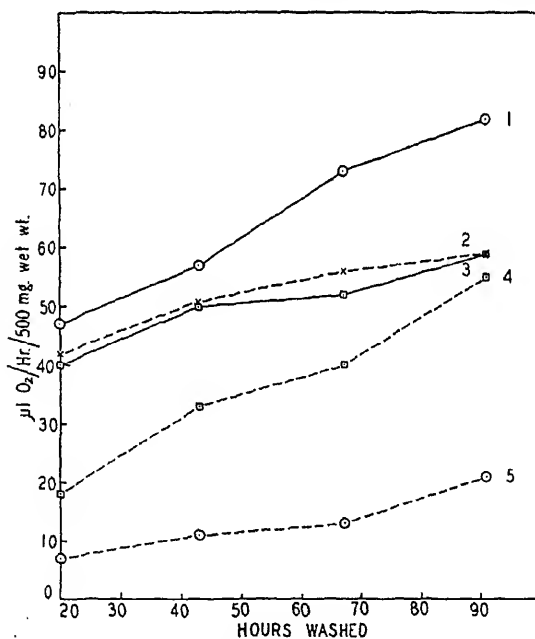


FIG. 3. Change in sensitivity of respiration to various agents as affected by length of washing period. Each point represents an average value obtained from duplicate vessels containing 25 potato slices in 3 ml. $M/100$ phosphate buffer, pH 6.1. Curves represent respiration in: (1) Air. (2) 5% O_2 -95% N_2 . (3) 5% O_2 -95% CO in the light. (4) 5% O_2 -95% CO in the dark. (5) Air in presence of $10^{-3} M$ KCN.

potato. The activity of enzyme X, and its relative participation in respiration, on the other hand, increases with longer washing time.

If the foregoing hypothesis is to be tenable, the sensitivity of potato respiration to carbon monoxide should decrease with increased washing time, since, in the preceding paper (1), it was shown that the action of enzyme X in tissue homogenates is insensitive to CO. Experiments were carried out to determine whether or not this prediction would be realized. Large batches of potato slices were prepared and washed in running tap water at 14°C. for 91 hr. Slices were removed at intervals, and divided into 2 groups, as before. With one group, the endogenous respiration was determined in air, in 95% N₂-5% O₂, in 95% CO-5% O₂ in the light and in the dark, and in air in the presence of 10⁻³ M KCN.

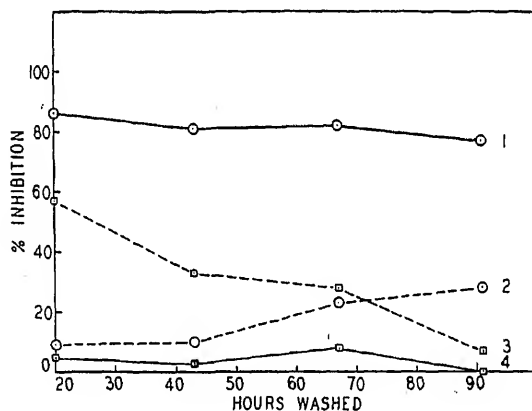


FIG. 4. Inhibition of slice respiration by various agents as a function of the length of washing period. Curves represent per cent inhibition by: (1) 1×10^{-3} M KCN vs. air. (2) 5% O₂-95% N₂ vs. air. (3) 5% O₂-95% CO in the dark vs. 5% O₂-95% N₂. (4) 5% O₂-95% CO in the light vs. 5% O₂-95% N₂.

The results obtained are graphically summarized in Figs 3 and 4. It is apparent from these curves that the inhibition of the respiration by CO in the dark, after 20 hr. washing, was 57%, while, after 91 hr., it was 7%. Throughout this period, the inhibition was largely reversible by light. Over the same interval of time, there was, as anticipated, a shift in the decrease of respiration from 9% to 28% consequent to the lowering of the pO₂ from 0.2 to 0.05 atm. The inhibition by cyanide remained high throughout the period of observation.

The marked rise in respiratory rate (Figs. 1 and 3) found on prolonged washing of the tissue at 14°C. was also observed when potato slices were incubated for several hours at 31°C. in *M*/100 phosphate buffer³ (2). Table I summarizes the hourly data obtained when the respiration of 25 potato slices (500 mg. wet weight washed in 14°C. tap water for 2 hr.) in 3.0 cc. *M*/100 phosphate buffer, pH 6.1, was measured for 8 hr. Each value is the average of quadruplicate determinations with the value of any individual determination not differing from the mean by more than 3%. In addition to the greater than 100% increase in oxygen consumption by the potato tissue over the 8 hr. experimental period, it should be noted that the R. Q. remained constant at approximately 1.0 after the first 4 hr., during which there was an increase from an initial value of 0.87. Preliminary observations indicated that, under similar experimental conditions, the sensitivity of the respiration to CO decreased with the time of incubation at 31°C., while its sensitivity to lowered pO₂ increased.

TABLE I
Increase of O₂ Consumption and CO₂ Production by Slices with Time at 31°C.

Hr.	O ₂ /Hr.	CO ₂ /Hr.	R. Q.
	$\mu\text{L.}$	$\mu\text{L.}$	
1	33.75	29.40	0.872
2	37.50	33.50	0.895
3	40.25	37.20	0.925
4	44.50	43.75	0.983
5	53.00	50.60	0.955
6	59.10	60.30	1.020
7	64.60	67.10	1.040
8	72.60	75.20	1.030

Homogenates were prepared from the other group of the slices used to obtain the data shown in Figs. 3 and 4. The activity of the homogenates on ascorbate was determined in air, in 95% N₂-5% O₂, and in 95% CO-5% O₂ in the light and in the dark. The data are presented in Fig. 5, while Fig. 6 shows the percentage decrease in homogenate action caused by lowering the pO₂ from 0.2 to 0.05 atm., as well as the inhibition caused by 95% CO-5% O₂ compared to 95% N₂-5% O₂.

Fig. 5 shows that there was a very great increase in the homogenate action on ascorbate in air over the 91 hr. washing time. During this period, the homogenate action on ascorbate, as evident from Fig. 6, became slightly more sensitive to lowered oxygen pressure and some-

³ A similar rapid rise was noted also when the slices were incubated in aerated tap water at 31°C.

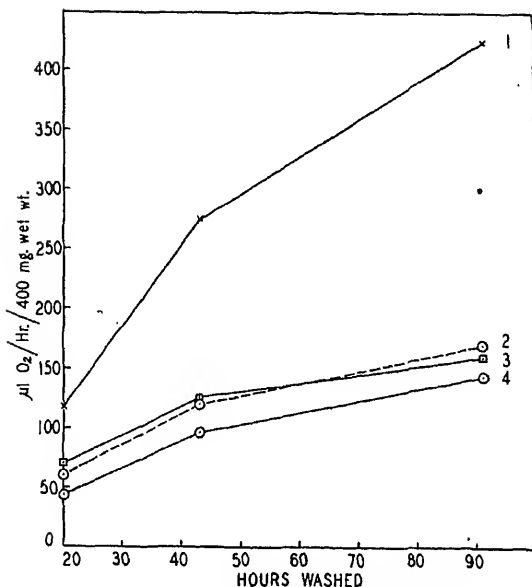


FIG. 5. Oxidation of ascorbate by potato homogenates in different gas mixtures as a function of length of washing period. Each point represents an average value obtained from duplicate vessels. Each vessel contained 1 ml. of homogenate (400 mg. wet weight of tissue), and 2 ml. *M/10* phosphate buffer, pH 6.1 in main room; 5 mg. ascorbate in 0.4 ml. H_2O , pH 6.0 in side arm; 0.2 ml. 20% KOH in midwell. Curves represent oxygen consumption in: (1) Air. (2) 5% O_2 -95% N_2 . (3) 5% O_2 -95% CO in the light. (4) 5% O_2 -95% CO in the dark.

what less sensitive to carbon monoxide. At all times the CO inhibition was significantly reversible by light.⁴

The evidence obtained with CO in the light and in the dark suggests that, when ascorbate is the substrate, potato homogenates show cytochrome oxidase activity even without added cytochrome c. The decreasing sensitivity to CO, as shown in Fig. 6, would then be indicative of a cytochrome oxidase activity in the homogenates which decreased with increased washing time, particularly over the first 40 hr. This, indeed, is what was observed from the direct enzyme assay (Fig. 2). The increased sensitivity, with time, of homogenate action on ascorbate to

⁴ A duplicate experiment gave results similar in all details, except that no increase of inhibition by 95% CO-5% O_2 in the light was observed over the entire period of washing.

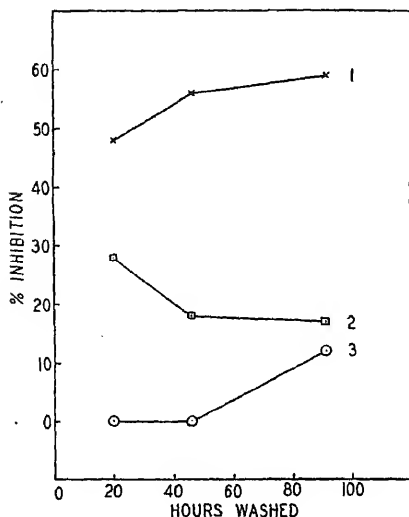


FIG. 6. Inhibition of homogenate oxidation of ascorbate by various agents as a function of length of washing period. Curves represent per cent inhibition by: (1) 5% O_2 -95% N_2 vs. air. (2) 5% O_2 -95% CO in the dark vs. 5% O_2 -95% N_2 . (3) 5% O_2 -95% CO in the light vs. 5% O_2 -95% N_2 .

lowered pO_2 would be expected from the greatly increased participation of the pO_2 sensitive enzyme X.

Comparison of the data shown in Figs. 3 and 4 with those of Figs. 5 and 6 demonstrates again the parallelism, as the washing period is lengthened, between the increasing sensitivity of the respiration of potato tissue to lowered pO_2 and the increasing activity of enzyme X in homogenates.

DISCUSSION

In the light of the changes which have been shown to occur in the respiration of potato tissue with washing, it is of interest to speculate on the terminal oxidase pattern of the intact tuber. Since the tuber depends mainly upon diffusion for its supply of oxygen, the pO_2 in the potato tissue is probably of such a low value that any terminal oxidase which is highly sensitive to lowered partial pressures of oxygen would be inactive. Under these conditions, one would anticipate that the respiration of the intact tuber would be mediated almost exclusively by

cytochrome oxidase rather than by some enzyme with the characteristics of enzyme X. The data presented lend support to this concept. By extrapolation back to zero time of the sensitivity of slice respiration to lowered pO_2 (Fig. 4), it is clear that the respiration of slices immediately prepared from the potato is unaffected by oxygen pressures as low as 0.05 atm. Further, if one extrapolates to zero time the percent inhibition of respiration by CO in the dark (Fig. 4), one finds an inhibition of about 70%. This is approximately the expected value if all the respiration goes through cytochrome oxidase, provided potato cytochrome oxidase has a relative affinity constant for CO and O_2 of about 10, as has yeast cytochrome oxidase. It appears not unreasonable to suggest, therefore, that, in the intact tuber, a major share of the respiration goes through cytochrome oxidase.

SUMMARY

The results presented in this paper give evidence for the participation of two terminal oxidases in the respiration of slices of white potato. One of these oxidases is photoreversibly inhibited by CO and relatively insensitive to lowered pO_2 (cytochrome oxidase), while the other is insensitive to CO but sensitive to lowered pO_2 . With increased washing time, the participation of cytochrome oxidase decreases while that of the pO_2 sensitive system increases. From a consideration of the characteristics of these terminal oxidases and the course of the shift in their participation in the respiration of washed potato slices, it is suggested that the respiration of the intact tuber is mediated primarily by cytochrome oxidase.

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New Reactions of Citrinin

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INTRODUCTION

Several years ago it was found in this laboratory that citrinin couples with diazotized *p*-nitroaniline in sodium carbonate or in methanolic solution. A similar observation has been published by Gore, Panse and Venkataraman (1) who used diazotized chloroanilines. Such a behavior could not be easily reconciled with the unusual quinonoid formula I, assigned to citrinin by Coyne, Raistrick and Robinson (2). In a recent publication Gore, Panse and Venkataraman (3) suggested structure II based on work earlier published by Cram (4) which would account for the coupling reaction. The further study of citrinin which was undertaken in this laboratory could not be completed, but the results are presented since some of the substances obtained in the course of this investigation may be of value in the elucidation of the structure of citrinin.

When a solution of citrinin in aqueous alkali was treated at room temperature with Raney nickel-aluminum alloy (5) a white crystalline reduction product III was isolated in good yield. The same product was obtained by Dr. Hershberg in this laboratory by catalytic reduction. Hetherington and Raistrick (6) isolated a different white crystalline reduction product using zinc in a glacial acetic acid-methanol solution. A comparison of the two compounds is shown in the following table:

	Product of Hetherington and Raistrick	Our product
M.P. (°C.)	About 240	169-170
M.P. of diacetate (°C.)	322-323	145-146
Stability of aqueous solution in air	Spontaneously and rapidly oxidized to citrinin	Stable
Color with aqueous FeCl ₃	Blue turning to brown with excess	Blue

Aside from the melting points the principal difference between the two reduction products is the marked contrast in oxidizability by atmospheric oxygen. The product of Hetherington and Raistrick was so rapidly oxidized in air that they did not attempt to isolate it as such but characterized it as the diacetate. Our product is stable and solutions show no tendency to assume the yellow color characteristic of citrinin, although it is oxidized to citrinin by a solution of bromine in chloroform. It was converted to a crystalline diacetate, a monomethyl ester, and an amide. Like citrinin itself, the reduction product coupled with diazotized *p*-nitroaniline.

The ease of reconversion of our reduction product into citrinin and the analyses of the compound and of its derivatives support the assumption that a benzene derivative is formed in the reduction of citrinin through the addition of two hydrogen atoms across the quinonoid system. Formula III is in agreement with Cram's findings and the blue color formed with FeCl_3 . The reduction product appears to be a substituted resorcinol.

A well crystallized substance which did not react with dinitrophenylhydrazine was obtained by oxidation of the acetate of the reduction product with chromic acid in acetic acid solution. The analysis of this product differed from that of the starting material by the loss of two hydrogen atoms and the addition of an oxygen atom. Formula IV is given tentatively to this substance although formula IVa is not excluded.

When a solution of citrinin in pyridine was treated with benzoyl chloride, the original yellow color soon diminished and after standing overnight the reaction mixture had changed to a thick, pale yellow, crystalline magma. A white compound was isolated which could only be purified by reprecipitation and the analysis supports its formulation as a tribenzoyl derivative of a citrinin in which the hetero ring is opened by the addition of water. It is possible that the parent substance, V, of this tribenzoate is present in the solution which is obtained when citrinin is dissolved in dilute alkali and left for some time at room temperature.

Upon refluxing citrinin with aqueous ammonium hydroxide it is converted into a substance which is apparently identical with the optically active product of the acid hydrolysis of citrinin, called product A by Hetherington and Raistrick (6). This substance gives a dibromo derivative in agreement with the observation of Gore *et al.* (3), who

found that product A couples with two moles of benzenediazonium-chloride.

From a consideration of all available evidence, citrinin appears to be the anhydro derivative of an enolized *p*-hydroxybenzoylformic acid. Similar compounds have been studied recently by Hunsberger and Amstutz (7).

EXPERIMENTAL¹

Citrinin was prepared by a method essentially the same as that of Hetherington and Raistrick (6) using corn steep liquor in the culture broth. The product was isolated by acidifying the filtered culture broth with 5 *N* H₂SO₄ to a pH of about 2, followed by extraction of the broth with $\frac{1}{4}$ its volume of chloroform and evaporation of the filtered chloroform *in vacuo* to dryness. The orange-brown residue was dissolved in hot absolute ethanol and filtered. Yellow crystalline citrinin, with a melting point ranging from 166° to 170° C., was obtained in average yields of 1 g./l. of broth by chilling the ethanolic solution. Repeated recrystallization from absolute alcohol raised this melting point to 171–172.5° always with decomposition. Its analysis agreed with that previously reported. Anal.: Calc'd for C₁₃H₁₄O₆: C, 62.37; H, 5.64. Found: C, 62.26; H, 5.86.

1. Dihydrocitrinin (III)

a. Reduction with Raney Alloy. A solution of 20 g. of citrinin in 450 ml. of 0.5 *N* NaOH was maintained at room temperature in a bath and 40 g. of Raney nickel-aluminum alloy was added gradually over a 15 min. period with occasional shaking of the mixture. Sodium hydroxide (100 ml. of 1 *N*) was added and the mixture was allowed to stand another hour at room temperature with occasional shaking. The mixture was diluted with 2 l. of water, filtered, acidified to Congo red with dilute H₂SO₄, and extracted 4 times with 400 ml. portions of ether. The ether was dried and evaporated, and the residue crystallized upon stirring with 25 ml. of benzene and 300 ml. of petroleum ether. The yield of product was 7.1 g. This material was dissolved in 100 ml. of hot chloroform and filtered. After cooling, 500 ml. of petroleum ether was added to it with stirring. After seeding and further chilling, the product slowly separated as white crystals with no tendency to become yellow on standing. M.p. 169–170°. Further recrystallization did not change this melting point. Anal.: Calc'd for C₁₃H₁₆O₆: C, 61.95; H, 6.40. Found: C, 62.06; H, 6.15. This product is only slightly soluble in water but is easily soluble on addition of sodium bicarbonate. It gives a blue color with a solution of ferric chloride.

b. Catalytic Reduction. (Experiment by Dr. E. B. Hershberg.) To 2 g. of citrinin dissolved in 10 ml. of 1.0 *N* KOH solution was added 1.5 g. of 10% palladium on charcoal catalyst. One mole of hydrogen was absorbed in 6 hr., after which the catalyst was removed by filtration and the aqueous solution was acidified to Congo red with dilute HCl. After extraction with ether, the ether solution was evaporated to dryness and the residue was crystallized from acetone-ligroin. There was obtained

¹ All melting points are corrected and were made by capillary tube method.

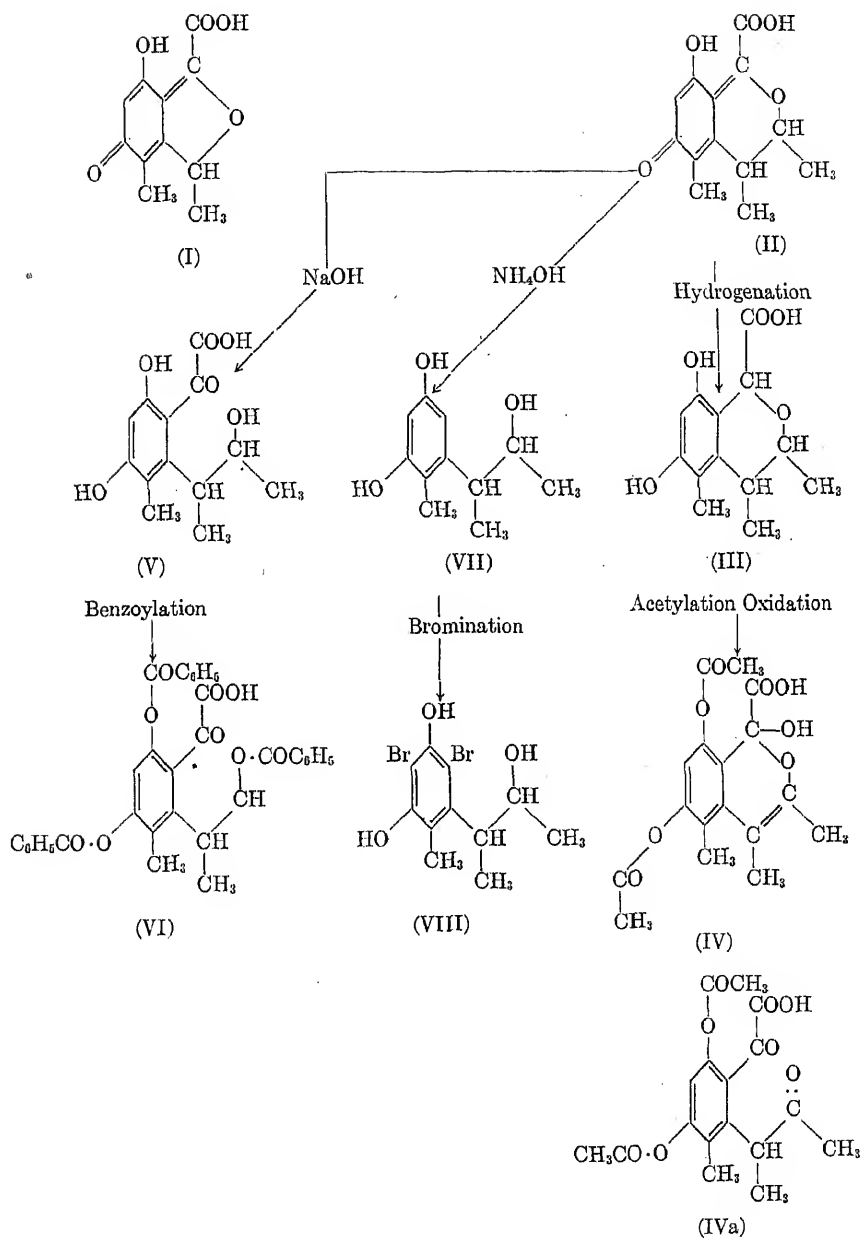


FIG. 1.

1.3 g. of product, m.p. 167–168°. After recrystallization from ethanol, the dihydrocitrinin melted at 171–172°. The substance gave no depression of the melting point with the product described above.

2. Dihydrocitrinin Diacetate

Dihydrocitrinin (2 g.) was dissolved in a mixture of 4 ml. of pyridine and 15 ml. of acetic anhydride. After standing at room temperature for 18 hr., and then heating in a bath at 75° for 1 hr., the solvent was evaporated *in vacuo* and the oily residue was stirred with 30 ml. of water containing 2 ml. of methanol. After cooling to 0°C., a white crystalline product separated. It was recrystallized once from 35 ml. of hot 40% methanol and twice from 25 ml. of hot benzene by addition of half a volume of petroleum ether. The yield was 1.2 g. of white crystals melting at 145–146°. Anal.: Calc'd for $C_{17}H_{20}O_7$: C, 60.69; H, 6.00. Found: C, 60.70; H, 6.10.

3. Dihydrocitrinin Methylester

Six g. of the reduction product were dissolved in 100 ml. of chloroform and to this was added 600 cc. of chloroform containing 90 millimoles of diazomethane. After standing 16 hr., the solution was evaporated to dryness *in vacuo*, the residue was extracted with 150 ml. of petroleum ether and the resulting solution was filtered from a small insoluble residue and evaporated to about 15 ml. This solution was chilled in an ice bath. Scratching or seeding caused a white crystalline product to separate in a yield of 4.6 g. After two recrystallizations from petroleum ether as above, the product had a melting point of 59.5–60.5°. Anal.: Calc'd for $C_{14}H_{18}O_5$: C, 63.10; H, 6.82; OCH_3 , 11.65. Found: C, 62.96; H, 6.56; OCH_3 , 11.86. This methyl ester on being heated for 1 hr. at 75° in a 50% methanol solution of sodium carbonate regenerated the original reduction product which was isolated, recrystallized and identified by melting point and mixed melting point.

4. Dihydrocitrinin Amide

The methyl ester of the reduction product (1.06 g.) was dissolved in 30 ml. of methanol and saturated with ammonia gas. After standing for 16 hr. it was evaporated to dryness and the residue was crystallized from a mixture of 40 ml. of water and 30 ml. of methanol. A white crystalline product was obtained in a yield of 0.8 g. Upon recrystallization from hot 50% methanol, it melted at 202–203°C. Anal.: Calc'd for $C_{13}H_{17}O_4N$: C, 62.15; H, 6.83; N, 5.58. Found: C, 61.54; H, 6.44; N, 5.44.

5. Dehydrogenation of Dihydrocitrinin (III) with Bromine

Half a g. of citrinin reduction product (2 mM) was dissolved in 20 ml. of chloroform and 0.3 ml. of bromine (5–6 mM) was added, whereupon a yellow oil separated. The solution was allowed to stand at room temperature for 1 hr., after which it was evaporated *in vacuo* at low temperature. The viscous, yellow residue was dissolved in 15 ml. of warm chloroform and 50 ml. of petroleum ether was added. A precipitate formed which was partly oily and partly crystalline. After chilling, this was filtered

off and the product was twice recrystallized from 5–10 ml. of chloroform by adding 50 ml. of petroleum ether. The final bright yellow crystalline product weighed 0.290 g. and melted at 172–173° with decomposition. Mixed melting point with citrinin did not show a depression.

6. Oxidation of Dihydrocitrinin Acetate (IV)

A 1.66 g. portion of the diacetate (0.005 *M*) was dissolved in 25 ml. of glacial acetic acid. This solution was cooled and to it was added a solution of 1.3 g. of chromium trioxide (corresponding to 4 oxygen atoms) in 1 cc. of water and 10 cc. of glacial acetic acid. The mixture was left overnight at room temperature; then the green solution was diluted with water and thoroughly extracted with ether. The ether extracts were washed 3 times with saturated NaCl solution and evaporated. The still adhering acetic acid was removed under vacuum and the residue which remained, 1.7 g., crystallized upon adding ether. It was recrystallized from benzene and petroleum ether. After further recrystallization from dilute acetone, the compound was obtained in the form of white crystals which melted at 177.5–178°C. The mixed m.p. with the starting material was 150–159°C. Anal.: Calc'd for $C_{17}H_{18}O_8$: C, 58.25; H, 5.18. Found: C, 58.43; H, 5.00. This product was hydrolyzed by boiling with dilute NaOH solution for 2 hr. Crystals were obtained after acidification and extraction with ether. Upon recrystallization from benzene-petroleum ether mixture the product melted at 239.9–240.9°, gave a purple coloration with a solution of $FeCl_3$ and coupled with diazotized *p*-nitroaniline. Unfortunately, there was not enough of the substance for analysis.

7. Benzoyl Derivative of Citrinin (VI)

A 2.46 g. portion of citrinin (0.01 *M*) was dissolved in 15 cc. of pyridine and 7 g. of benzoyl chloride (0.05 *M*) was added. The solution was left overnight, after which it appeared as a whitish magma. It was then poured into a mixture of ice and H_2SO_4 and the gummy precipitate was washed by decantation. The residue was taken up in ether, washed thoroughly with water, and dried. The ether solution was poured into petroleum ether and gave a flocculent white precipitate which was filtered and washed with petroleum ether. The material could not be crystallized from other solvents and therefore was once more reprecipitated from ether with petroleum ether and then analyzed. The substance sintered at 112°C. and was completely melted at 136°C. Anal.: Calc'd for $C_{34}H_{28}O_9$: C, 70.32; H, 4.86. Found: C, 69.79; H, 5.09. On slight heating of this substance with NaOH solution, the solution became yellow, and upon acidification gave citrinin.

8. Reaction of Citrinin with Ammonia (VII)

When citrinin (5 g.) was heated with a mixture of 50 ml. of water and 50 ml. of concentrated NH_4OH solution under reflux in a bath of 70°C. for 5 hr., a dark red-brown solution resulted, which, on acidification, evolved CO_2 . Some amorphous deposit was filtered off, the solution was extracted with ether, the ether evaporated, and the residue crystallized twice by dissolving it in ether and adding petroleum ether.

Yield 3.0 g. After 2 more recrystallizations from chloroform with addition of petroleum ether to saturation, the white nacreous crystals had a melting point of 127–128.5°C., and their analysis agreed with the product A of Hetherington and Raistrick (6) obtained by acid hydrolysis of citrinin. Anal.: Calc'd for $C_{11}H_{14}O_3$: C, 67.34; H, 8.22. Found: C, 67.52; H, 8.22. A 1% solution in methanol in a 10 cm. tube $[\alpha]_D = -34.0^\circ$. Hetherington and Raistrick (6) obtained a melting point of 128–130°C. and a rotation of the green mercury line of -43.7° .

9. Dibromo Derivative (VIII) of Substance VII

One-half g. of product VII was suspended in 10 ml. of chloroform and 5 ml. of Br_2 added. Fumes of HBr appeared. The chloroform was evaporated after 1 hr. and the product was crystallized from its solution in 5 ml. of benzene by addition of 50 ml. of petroleum ether. These crystals were extracted with 250 ml. of boiling water and the aqueous solution evaporated *in vacuo* to about 20 ml. and chilled, whereupon it deposited dense white crystals. These were dissolved in 250 ml. of boiling petroleum ether, the solution was evaporated to 50 ml. and chilled. White crystals were obtained in a yield of 0.26 g. melting at 128–129°C. Anal.: Calc'd for $C_{11}H_{14}O_3Br_2$: C, 37.29; H, 3.98; Br, 45.2. Found: C, 38.14; H, 4.10; Br, 45.45.

SUMMARY

A new reduction product of citrinin is described, together with several derivatives.

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Amino Acid and Unsaturated Fatty Acid Requirements of *Clostridium sporogenes*¹

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INTRODUCTION

In a previous publication (1) it was shown that moderate but continuous growth of a number of strains of *Clostridium sporogenes* could be obtained on a chemically defined medium containing amino acids, glucose, biotin, *p*-aminobenzoic acid, nicotinic acid, salts, buffer, and sodium thioglycolate. In that paper data were also presented which indicated that (a) an unidentified factor present in partial protein digests was necessary for heavy growth of *Cl. sporogenes* and (b) oleic acid was effective in replacing biotin in the basal medium.

In the present paper the results of further research along these two lines are presented. Data are given which show that heavy growth of the test organism can be obtained by the addition, in suitable concentrations, of certain combinations of amino acids such as arginine, tyrosine, phenylalanine, and tryptophan. Evidence is presented that these amino acids are used as substrates by the organism, even in the presence of glucose.

Oleic, vaccenic, linoleic, and ricinoleic acids are shown to be effective in replacing biotin in the basal medium if a suitable emulsifying agent is also included.

EXPERIMENTAL

Cultures, Media and Techniques

The cultures, methods of preparing inoculum, and conditions of fermentation were the same as previously described (1). For routine work American Type Culture No.

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10,000 was used. This is the culture employed by Knight and Fildes (2) and carried by The National Collection of Type Cultures, London, England, under the number 533. Another transfer of this culture was obtained from Dr. L. S. McClung, Indiana University, and is listed in this paper as No. 533. Transfers of the other cultures were obtained from Dr. Elizabeth McCoy of our Agricultural Bacteriology Department.

Two media were used. Medium P has the following composition: Norit-treated, acid-hydrolyzed casein 0.5%, glucose 1.0%, sodium thioglycolate 0.1%, K_2HPO_4 0.7%, biotin 40 $m\gamma$ /100 ml., *p*-aminobenzoic acid 10 γ /100 ml., nicotinic acid 60 γ /100 ml., L-tryptophan 0.004%, L-cystine 0.005%, salt solution B 0.5 ml./100 ml. Salt solution B contains $MgSO_4 \cdot 7H_2O$, 10 g.; NaCl, 0.5 g.; $FeSO_4 \cdot 7H_2O$, 0.5 g.; and $MnSO_4 \cdot 4H_2O$, 0.5 g.; dissolved in 250 ml. of H_2O .

Medium P-A is the same as P, except that a mixture of amino acids corresponding to that in casein (3) was used instead of the casein hydrolyzate. This medium contained 10 ml. of the following amino acid mixture/100 ml.:

L-arginine hydrochloride	0.240 g.
L-histidine hydrochloride	0.145
DL-lysine hydrochloride	0.850
L-tyrosine	0.320
DL-tryptophan	0.180
DL-phenylalanine	0.520
L-cystine	0.018
DL-methionine	0.350
DL-serine	0.650
DL-threonine	0.400
DL-leucine	1.210
DL-isoleucine	0.650
DL-valine	0.700
DL-glutamic acid	2.280
DL-aspartic acid	0.630
glycine	0.025
DL-alanine	0.560
L-proline	0.410
L-hydroxyproline	0.100

H_2O to make 100 ml.

RESULTS

Response to Amino Acids

In an attempt to isolate the unidentified growth-promoting factor from partial protein digests, it was noted that a portion of the activity accompanied the basic amino acid fraction. Accordingly arginine, lysine, and histidine at 0.5% levels were included one at a time in the assay series to determine whether the amino acids themselves in high concentrations might give increased growth. Arginine had a definite

stimulatory effect, while lysine and histidine were either ineffective or provided very little stimulation. For example, without arginine the galvanometer reading of the Evelyn colorimeter was 70, and with either 50 or 100 mg. of arginine the reading was 60.

The discovery that arginine was stimulatory was surprising in view of the fact that the basal medium contained 0.5% hydrolyzed casein, which might be expected to supply an adequate quantity of arginine (2 mg./10 ml.) for the growth of the organism. Since a high concentration of arginine was beneficial to the growth of the organism, it was of interest to see whether any other amino acids would produce a stimulation when added alone to the basal medium, and also when added in the presence of a high concentration of arginine. When 25 mg./10 ml. of the L-isomers (or 50 mg. of the DL-compounds) of 19 amino acids were added separately to the basal medium, arginine, tryptophan, and tyrosine were found to produce the greatest stimulation. Several other amino acids produced a slight stimulation, but the above-mentioned 3 amino acids were definitely superior in this respect. The galvanometer readings of these compared with the control in one experiment were as follows: control 73, arginine 53, tryptophan 53, and tyrosine 60. In the presence of L-arginine hydrochloride, however, phenylalanine and tyrosine were markedly stimulatory, tryptophan and proline moderately so, and cystine slightly stimulatory. Recrystallization of L-proline removed its stimulatory effect completely, but two recrystallizations of L-tryptophan resulted in no decrease in activity. Synthetic DL-tryptophan also appeared to possess about equal activity when used at twice the concentration of that of the L-form. L-cystine appeared to give a slight stimulatory effect, even after recrystallization.

Many experiments were performed to determine the best levels of the 4 most stimulatory amino acids, alone and in various combinations. Table I shows some typical results. Arginine was by far the most effective single addition to the basal medium. The most potent supplement to arginine was tyrosine; phenylalanine came next and tryptophan gave the least stimulus. Nearly optimal growth was obtained with 25 mg. each of arginine and tyrosine. Doubling the amounts of these acids gave only a slight additional increase in growth. No further growth was promoted by the addition of a third or fourth amino acid to the arginine-tyrosine combination. In fact, a small but definite inhibitory effect was usually noted when tryptophan was used with arginine and

TABLE I

*Effect of High Levels of Arginine, Tryptophan, Phenylalanine, and Tyrosine,
Alone and in Combinations (Medium P)*

L-Arginine · HCl	DL-Tryptophan	DL-Phenylalanine	L-Tyrosine	Galv. read.
<i>mg./10 ml.</i>	<i>mg./10 ml.</i>	<i>mg./10 ml.</i>	<i>mg./10 ml.</i>	
0	0	0	0	76
25	0	0	0	67
50	0	0	0	66
0	25	0	0	77
0	50	0	0	75
0	0	25	0	79
0	0	50	0	78
0	0	0	25	74
0	0	0	50	70
25	25	0	0	66
25	50	0	0	63
25	0	25	0	44
25	0	50	0	41
25	0	0	25	29
25	0	0	50	25
25	25	25	0	35
25	25	0	25	32
25	0	25	25	31
25	25	25	25	31
50	25	0	0	62
50	50	0	0	60
50	0	25	0	39
50	0	50	0	32
50	0	0	25	27
50	0	0	50	23
50	50	50	0	40
50	50	0	50	25
50	0	50	50	30
50	50	50	50	65
0	50	50	0	71
0	50	0	25	67

either of the other two amino acids. Inhibition was also shown by high levels of all 4 amino acids taken together.

That the stimulatory effect of arginine and tyrosine was not due to impurities was indicated by retention of full activity after repeated crystallizations.

The data of Table II indicate that alone, lysine, histidine and octopine have little or no activity, but in combination with tyrosine the effect is somewhat more definite; none of them, however, compares with arginine in potency. On the other hand, ornithine, as might be expected from its structural and physiological relationships to arginine, did serve as a substitute for the latter. On a molar basis they are practically equal.

TABLE II

Effects of the Basic Amino Acids Alone and with Tyrosine in Medium P

Basic amino acid		Tyrosine	Galv. read.
Name	Amount		
—	mg./10 ml.	mg./10 ml.	
—	None	None	72
—	None	20	70
L-Arginine HCl	25	None	59
L-Arginine HCl	25	20	27
DL-Lysine HCl	50	None	69
DL-Lysine HCl	50	20	61
L-Histidine HCl	25	None	72
L-Histidine HCl	25	20	64
DL-Ornithine HCl	40	None	64
DL-Ornithine HCl	40	20	33
Octopine	20	None	71
Octopine	20	20	64

To determine whether the growth stimulation by certain amino acids was a general phenomenon among the different strains of *Cl. sporogenes*, 8 strains of this organism, of which two, No. 10,000 and No. 533, originated from the parent strain N.C.T.C. No. 533, were tested for their response to L-arginine hydrochloride and L-tyrosine on medium P and on medium P-A, a chemically defined medium. These results are presented in Table III. All of the strains were markedly stimulated by the addition of these two amino acids to either medium. It can be seen that a medium containing crystalline amino acids in the proportions in which they occur in casein and equivalent to 0.5% casein is suitable for good growth of the organism if it is supplemented by high concentrations of arginine and tyrosine. If the mixture of amino acids in medium P-A is replaced by the combination of 16 amino acids used by Fildes

TABLE III

Response of Various Strains to High Concentrations of Arginine and Tyrosine on a Chemically defined Medium and on the Basal Medium

Culture	Galvanometer readings			
	Medium P		Medium P-A	
	No supplement	0.50% arginine 0.25% tyrosine	No supplement	0.50% arginine 0.25% tyrosine
No. 10,000	76	31	84	32
No. 533	72	31	77	45
No. 44	80	36	79	40
No. H-2	90	49	96	57
No. 12	69	23	74	29
No. 1	77	30	79	28
No. 4	88	37	89	39
<i>Cl. parasporogenes</i>	73	29	76	49

and Richardson (4), and isoleucine is added (1), the total amino acid concentration is only one-sixth that in medium P-A. On this lean basal medium the addition of 50 mg. each of arginine and phenylalanine gave only fair stimulation, indicated by a galvanometer reading of 76 compared to a control of 87.

Activity of Partial Protein Digests

In the previous publication (1) the marked stimulatory effect of partial protein digests on the growth of the organism was demonstrated. From the data presented above it would appear that a considerable portion of the stimulatory effect of partial protein digests on the growth of *Cl. sporogenes* might be due to the free arginine, tyrosine, and phenylalanine present in such digests. It was of interest to determine whether the total activity of the digests might be attributed to their content of these amino acids. For this experiment, partial digests of casein and fibrin were assayed at different levels so that curves could be plotted for growth *vs.* amount of protein digest added. Simultaneously, turbidities were obtained for various combinations of arginine, tyrosine, and phenylalanine. All amino acids were added in the proportions found in the two proteins. (Block and Bolling (3) give the following percentages for arginine, tyrosine, and phenylalanine, respectively: casein 4.1,

6.4, 5.2; fibrin 6.8, 5.1, 7.0.) The turbidities given by the amino acids were read on the assay curves to obtain the amounts of protein digests to which they were equivalent. For example, in the first experiment 3.5 mg. of L-arginine hydrochloride, 2.5 mg. of L-tyrosine and 7.0 mg. of DL-phenylalanine, which are the quantities of these amino acids calculated as being present in 1.0 ml. of the fibrin hydrolyzate, produced a growth equivalent to that produced by only 0.258 ml. of fibrin hydrolyzate. Therefore, the fibrin hydrolyzate was 3.88 times as potent as the corresponding concentrations of arginine, tyrosine, and phenylalanine contained in it; or its activity ratio may be said to be 3.88. In Table IV these data are given. Fibrin was approximately 4 times as

TABLE IV
*Activity of Fibrin and Casein Digests Related to Their Content
of Three Amino Acids*

Amino acids added to medium P			Activity			
L-Argi- nine · HCl	L- Tyrosine	DL-Phenyl- alanine	Calculated		Obtained	Ratio ^c
			Fibrin ^a	Casein ^b		
mg./10 ml.	mg./10 ml.	mg./10 ml.	ml.	mg.		
3.5	2.5	7.0	1.0	—	0.258 ml.	3.88
10.5	7.5	21.0	3.0	—	0.760 ml.	3.95
3.0	4.9	7.5	—	75	47 mg.	1.59
4.0	6.5	10.0	—	100	53 mg.	1.89
6.0	9.8	15.0	—	150	75 mg.	2.00

^a This partial fibrin hydrolyzate (5%) was obtained from Dr. D. V. Frost, Abbott Laboratories, Inc., North Chicago, Ill.

^b Casitone, a pancreatic digest of casein and a product of Difco Laboratories, Inc., Detroit, Mich., was used here.

^c These values were obtained by dividing the figures in Column 4 or 5 by those in Column 6.

active as a corresponding amount of arginine, tyrosine, and phenylalanine, while casein was about twice as active as the amino acid mixtures. It might be assumed that the greater potency of the fibrin and casein digests was due to the amino acids other than arginine, tyrosine, and phenylalanine which these protein preparations contribute. However, no further stimulation was obtained if a mixture of all the amino acids (except arginine, tyrosine, and phenylalanine) in the proportions

in which they occur in casein was added to tubes containing the amino acid mixtures shown in Table IV.

The fact that amino acids in peptide form appear to be superior to the free amino acids was also corroborated by a comparison of the activity of partial protein digests before and after a drastic hydrolysis (25% H_2SO_4 at 120°C . for 17 hr.). When casein and fibrin digests were assayed before and after such treatment the turbidities induced by comparable concentrations were as follows: casein, before 48, 37; after 56, 48; fibrin, before 41, after 61. When a corresponding solution of crystalline amino acids was treated in a similar manner no decrease in growth stimulation was noted.

Effect of High Levels of Completely Hydrolyzed Proteins

It was found that increasing the concentration of hydrolyzed casein in the basal medium resulted in better growth of the organism. While this response was not linear above a 2% concentration, no inhibition resulted when as much as 4% of hydrolyzed casein was used. This effect could not be reproduced if a mixture of crystalline amino acids (in the proportions in which they occur in casein) was used instead of hydrolyzed casein. In this case an inhibition was noted. This inhibition could probably be attributed to the high concentration of D-amino acids in the solution, since 13 of the 19 crystalline amino acids used were of the DL-form.

To determine whether the stimulatory effect of high concentrations of hydrolyzed casein could be attributed to the higher levels of the 3

TABLE V
Comparison of Different Concentrations of Hydrolyzed Casein and Amino Acids on Growth of Cl. sporogenes in Medium P

Hydrolyzed casein	L-Arginine hydrochloride	L-Tyrosine	DL-Phenylalanine	Galv. read.
mg./10 ml.	mg./10 ml.	mg./10 ml.	mg./10 ml.	
50	None	None	None	71
100	None	None	None	56
200	None	None	None	44
50	2.0 ^a	3.2 ^a	5.2 ^a	58
50	6.0 ^a	9.6 ^a	15.6 ^a	46

^a The concentrations of the 3 amino acids used in this experiment are the quantities that 50 mg. and 150 mg. of hydrolyzed casein/tube (10 ml.) would supply.

most stimulatory amino acids, an experiment was set up in which the growth response of the test organism on media containing 0.5, 1.0, and 2.0% hydrolyzed casein was compared with that obtained on media containing 0.5% hydrolyzed casein supplemented with the quantities of arginine, tyrosine, and phenylalanine which the high concentrations of casein would supply. The results of this experiment are presented in Table V. It can be seen from these data that virtually all of the stimulatory effect of the high concentrations of hydrolyzed casein can be attributed to the increase in concentration of the stimulatory amino acids and that the other amino acids have little effect.

Utilization of Arginine and Tyrosine as Fermentation Substrates

Since large quantities of the stimulatory amino acids were required for heavy growth of the organism, some knowledge of the function of these amino acids in the growth of the organism appeared desirable. Two theories which might account for the high concentrations required are: (a) The organism requires a peptide for good growth and the addition of the appropriate amino acids in high concentration stimulates formation of the peptide; (b) the stimulatory amino acids act as preferred substrates for the organism.

To test the validity of the second hypothesis experiments were set up, in which ammonia, glucose, arginine, and tyrosine were determined after fermentation. The results of these experiments are given in Table VI.

Glucose was determined with the Shaffer and Somogyi reagent No. 50 (5) in which 5.0 g. of KI/1000 ml. was used. Ammonia was determined by adding phosphate buffer to 10 ml. samples to a pH of 8.0, diluting to approximately 55 ml., and distilling about 35 ml. into a receiver containing 0.05 *N* H₂SO₄. The residual acid was titrated with 0.05 *N* NaOH. pH 8.0 was chosen in order to minimize the destruction of the arginine by the alkali. (Nichols and Foote (6) reported that no NH₃ was recovered from a solution of guanidine carbonate distilled at pH 8.0). As can be seen from the data in Table VI, Experiment I, the uninoculated arginine-tyrosine medium yielded only 0.13 mg. of NH₃ more than that obtained on the uninoculated basal medium. The amino acids were determined microbiologically. *Lactobacillus delbrückii* No. 3 was used for arginine and *Leuconostoc mesenteroides* No. PD-60 was used for tyrosine.

From the data presented in Table VI it is apparent that arginine and tyrosine were used as substrates by the organism, even in the presence of glucose. This is indicated directly by the disappearance of the amino

TABLE VI

Utilization of Amino Acids and Glucose by Cl. sporogenes No. 10,000 (Medium P)

Experiment no.	Before fermentation ^a				After fermentation					
	Glucose	Arginine	Tyrosine	Casitone	Galv. read.	pH	NH ₃	Glucose ^b	Arginine	Tyrosine
	mg./10 ml.	mg./10 ml.	mg./10 ml.	mg./10 ml.			mg./10 ml.	mg./10 ml.	mg./10 ml.	mg./10 ml.
1	100	None	None	None	—	—	0.89 ^c	—	—	—
	100	24	20	None	—	—	1.02 ^c	—	—	—
	100	None	None	None	69	6.0	1.46	95.2	—	—
	None	24	20	None	51	7.1	8.64	—	—	—
	100	24	20	None	31	6.2	5.74	68.0	—	—
	200	24	20	None	32	6.1	5.47	167.0	—	—
	100	None	None	150	35	5.9	2.76	61.0	—	—
2	100	21.2	19.8	None	28	—	—	—	1.09	0.2
	None	21.3	20.6	None	49	—	—	—	1.02	5.7

^a In Experiment I, the values given before fermentation were not obtained by analysis but represent only the quantities weighed out and added to the medium. In Experiment 2, the amino acids were determined before and after fermentation.

^b The thioglycolate in the basal medium gave increased values in the sugar determinations, e.g., there was a 107% recovery of the glucose in the uninoculated control. The maximum error in the determinations can probably be considered as 7%.

^c These tubes were not inoculated, but were sterilized and incubated in the same manner as the other tubes.

acids and indirectly by the quantity of NH₃ produced. While the glucose values are somewhat high, due to the interference of sodium thioglycolate in the determination, it is, nevertheless, evident that a large portion of the glucose is not fermented by the organism. In the presence of amino acids less than 50 mg./tube was fermented while without the amino acids only a negligible quantity was used.

Several other strains of *Cl. sporogenes* were tested and found to give results similar to A.T.C. No. 10,000, i.e., little utilization of glucose and the production of large quantities of NH₃ on the arginine-tyrosine medium.

Biotin Replacement by Unsaturated Fatty Acids

In the preceding paper (1) the ability of oleic acid to replace biotin in the basal medium was demonstrated. It was pointed out, however, that this replacement effect was not easily reproducible, unless a suitable emulsifying agent was incorporated in the medium.

Williams *et al.* (7) have shown that the use of Tween (fatty acid esters of polyoxyethylene derivatives of sorbitan, products of the Atlas Powder Company, Wilmington, Del.) eliminates the toxic action of oleic acid for different lactic acid bacteria. These investigators found that without Tweens the growth stimulation from oleic acid could be demonstrated only within a narrow pH range and limited concentrations while the addition to the basal medium of one of the Tweens (such as Tween 40, itself inactive) greatly extended the active pH range and acted as a "detoxifying agent" for the oleic acid. They also noted that Tween 80, which is an oleic acid ester, served as a convenient source of oleic acid in a non-toxic form for the lactic acid bacteria.

Two Tweens were tested in this investigation, Tween 40 and Tween 80. Tween 40 is a palmitic acid ester and Tween 80 is an oleic acid ester. The biotin-replacing effect of oleic, linoleic, ricinoleic, and vaccenic acids, and Tween 80 on the biotin-deficient basal medium and on a biotin-deficient medium containing arginine and tyrosine is shown in Table VII. It can be seen from these data that all these materials were

TABLE VII
Response of Cl. sporogenes to Unsaturated Fatty Acids on Biotin-Deficient Media

Medium	Additions/10 ml. ^a	Galv. readings
P	None	100
	4 mγ biotin	67
	30, 50, 70, 90, 110γ oleic acid	70, 72, 66, 66, 66
	30, 50, 70, 90, 110γ linoleic	76, 74, 75, 71, 68
	30, 50, 70, 90, 110γ ricinoleic	97, 93, 85, 81, 80
	30, 50, 70, 90, 110γ <i>cis</i> -vaccenic	67, 68, 71, 68, 67
P, with 25 mg. of arginine and 20 mg. of tyrosine/ 10 ml.	None	94
	4 mγ biotin	31
	60, 120γ oleic acid	42, 31
	60, 120γ linoleic	54, 46
	60, 120γ ricinoleic	93, 75
	60, 120γ <i>cis</i> -vaccenic	34, 31
	10, 20 mg. Tween 80	35, 31

^a 5 mg. of Tween 40/10 ml. were used with each of the fatty acids.

effective in replacing biotin for *Cl. sporogenes*. The arginine-tyrosine medium appeared to accentuate the differences in activity of the fatty acids. From these experiments, oleic and vaccenic acids have the same activity, linoleic somewhat less, and ricinoleic distinctly less than the other three.

Williams and Fieger (8) found that elaidic acid, the *trans*-form, was more effective than oleic acid, the *cis*-form, as a biotin replacement for

Lactobacillus casei. Our tests showed that elaidic acid was only about 30% as potent for *Cl. sporogenes* as was the naturally-occurring oleic acid. Furthermore, a synthetic preparation of *trans*-vaccenic acid gave only 25% of the growth stimulation obtained with synthetic *cis*-vaccenic acid. Natural vaccenic acid is generally considered to have the *trans*-configuration rather than the *cis*-type.

In the experiments described above, Tween 40 was used at a 5 mg./tube level, but a concentration as low as 500 γ /tube was found to be sufficient when 100 γ /tube of oleic acid was added.

Tween 40 exerted a surprising toxicity for the organism in the absence of any of the fatty acids. This toxicity was apparent when as little as 500 γ /tube of Tween 40 was added to the basal medium. The addition of varying concentrations of biotin was not effective in reversing the growth inhibition, so apparently Tween 40 did not exert a competitive inhibition with biotin (1).

DISCUSSION

Cl. sporogenes apparently belongs to that class of putrefactive anaerobes which can utilize either glucose or protein degradation products as carbon sources. Previous investigators (9,10,11) have noted that large quantities of NH_3 were produced by this organism on various complex media. In this investigation it has been demonstrated that the organism can be grown on a chemically defined medium and that large amounts of certain amino acids or peptides in the medium were very stimulatory. As with the more complex media containing meat infusions, growth on the simplified media also led to the production of large quantities of NH_3 .

The preferential utilization of particular amino acids, such as arginine, tyrosine, phenylalanine, and tryptophan, is not fully understood, but hydrogenation and dehydrogenation of certain amino acids in the manner described by Stickland and others (12,13,14) may be involved. It was found that the arginine and tyrosine added to the basal medium were either dissimilated or converted into products which were no longer active for the amino acid assay organisms (*L. delbrückii* and *L. mesenteroides*).

It appears that *Cl. sporogenes* can utilize a number of amino acids, but that certain of the amino acids are more effective, and are probably required in certain concentrations, before the other amino acids, are utilized. In this connection arginine was found to be particularly

active. While this amino acid alone, even in high concentrations, produced only moderate stimulation, combinations of it with tyrosine or phenylalanine produced very good growth.

For devising a chemically defined medium for optimum growth of the organism a number of possibilities in regard to amino acid combinations exist. A concentration of 0.2–0.5% L-arginine hydrochloride should be added to the basal medium (which should contain a solution of crystalline amino acids). As the second stimulatory amino acid, tyrosine or phenylalanine can be used. Tyrosine appears to be the more active, but it has the disadvantage of limited solubility. If it is added to the culture tubes as an acid solution, then each tube must be neutralized separately. The tyrosine precipitates on neutralization, but it was found to redissolve (in concentrations as high as 0.2%) upon autoclaving and to remain in solution thereafter. Phenylalanine can be used in place of tyrosine, but if the usual commercially available DL-preparation is used, twice as much is required.

The use of Tween greatly facilitated the demonstration of the biotin replacement effect of unsaturated fatty acids. Without the use of such emulsifying agents, the effect of the fatty acids is so erratic and hard to reproduce that an investigator might easily overlook the phenomenon.

Williams *et al.* (7) showed that the effect of oleic acid was not due to a contamination with biotin since the oleic acid stimulation was not reversible by the avidin in raw egg white as was the case with biotin stimulation. These workers further demonstrated that oleic acid or a similar compound was required by certain lactic acid bacteria even in the presence of biotin. In the experiments reported here, the Tween 40 was found to have a marked inhibitory effect on the growth of the organism on media containing biotin but no unsaturated fatty acid. This inhibition serves as additional evidence that the fatty acids themselves are active, and that the activity is not a result of a trace of biotin in either the Tween or the fatty acids, since the presence of biotin did not reverse the inhibition (1).

Williams *et al.* have postulated that oleic acid or a similar unsaturated fatty acid is a required growth factor for certain organisms, and that one of the functions of biotin is to serve in the synthesis of this acid. The results presented in this paper are in agreement with the work of these investigators and extend the scope to include an anaerobe and additional unsaturated fatty acids. In addition to oleic and linoleic acids found to be active by other workers, vaccenic and ricinoleic acids

were also found to be effective. Vaccenic acid has recently been reported active in the replacement of biotin for certain lactic acid bacteria by Axelrod *et al.* (15), but, to the authors' knowledge, this is the first instance in which ricinoleic acid has been found active in this respect. It was of particular interest in this investigation to test the activity of ricinoleic acid, $\text{CH}_3 \cdot (\text{CH}_2)_5 \cdot \text{CH}(\text{OH}) \cdot \text{CH}_2\text{CH}:\text{CH}(\text{CH}_2)_7 \cdot \text{COOH}$, since Pappenheimer (16) found his highly active concentrates of "sporogenes vitamin" contained an unsaturated hydroxy-acid with ten carbon atoms. Inasmuch as a number of C_{18} unsaturated fatty acids have been found to be effective in replacing biotin for *Cl. sporogenes*, the effect appears to be rather non-specific, and it seems quite possible that the unsaturated fatty acids with lower molecular weights might also be active. It also appears probable that an unsaturated hydroxy-acid, such as that described by Pappenheimer, exists in nature and has not yet been isolated in pure form. Such an acid would presumably be more active for *Cl. sporogenes* than the C_{18} acids if the activity of Pappenheimer's concentrates can be used as an index.

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SUMMARY

High concentrations of certain combinations of amino acids can replace partial protein digests in media for *Clostridium sporogenes*. Arginine or ornithine in combination with tyrosine or phenylalanine produces good growth of the organism, but partial protein digests appear to have a stimulatory effect above that due to their content of these amino acids. When arginine and tyrosine are included in the medium, they are utilized by the organism and large quantities of NH_3 are produced. Glucose does not exert a sparing action on the utilization of arginine and tyrosine, but in their presence more glucose is utilized and better growth results.

Oleic, vaccenic, linoleic, and ricinoleic acids are active, in the order named, in replacing biotin, both in the basal medium and in an arginine-tyrosine medium. The use of emulsifying agents such as the

Tweens greatly facilitates the demonstration of this phenomenon. Tween 80, an oleic acid ester, is active in high concentrations in replacing biotin.

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A New Method for the Colorimetric Determination of the Total Esterified Fatty Acids in Human Sera

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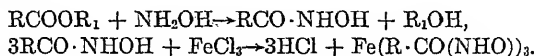
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Although several methods, including colorimetric (22,23), are available for determining serum fatty acids, most do not seem practical for routine clinical use because of the meticulous care required for accuracy in the analysis of small amounts of blood. To obviate these difficulties, a simple method is designed for colorimetric determination of the esterified fatty acid content² in sera.

The method is based upon the conversion of fatty acid esters into the corresponding hydroxamic acids by hydroxylamine hydrochloride and sodium hydroxide, and their subsequent conversion into colored ferric salts.

Jeanrenaud (1) in 1889 prepared benzoic hydroxamic acid from benzoic acid ester by means of hydroxylamine hydrochloride and sodium ethylate and remarked that the hydroxamic acid gives the characteristic iron chloride reaction. Later, Dieterle, Diester and Thimann (2) prepared stearic and palmitic hydroxamic acids from the corresponding methyl esters by the same general method and stated that these compounds gave the characteristic red violet color for hydroxamic acids. Feigl (3) developed this reaction as a qualitative spot test for esters of carboxylic acids and, with Anger and Frehden (4), studied its specificity. The reactions involved are given by Feigl as follows:



Employing these reactions, Hill (5,6) recently developed a method for the quantitative determination of certain lipides used on steel. When we tried the procedure developed by Hill on various quantities of ethyl stearate, the color developed was found to follow Beer's law by spectrophotometric analysis. However, when different

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² According to Davis (21), the proportion of free fatty acid to the total fatty acid content of human sera is small, calculated at approximately 3%.

amounts of alcohol-ether extract of blood serum were substituted for the ethyl stearate (after redissolving the lipides in absolute ether) the color development did not follow Beer's law. Instead, the curves had a sharp bend when plotted in the usual way. For this reason many of the conditions concerned with the development of the color were studied to find a satisfactory procedure for the colorimetric estimation of serum esterified fatty acids.

The reagent for color development as described originally by Hill contained ethyl alcohol, water, perchloric acid, and ferric perchlorate. Later (6), he recommended adding hydrogen peroxide or nitric acid to prevent the reduction of the ferric ions by the excess of hydroxylamine. It occurred to us that, if an excess of ferric ions was added, the presence of reducing agents and other interfering substances that may be present in serum might not alter color development. The results were not satisfactory, however, until suitable concentrations of water and perchloric acid were determined. In addition, ferric perchlorate was purchased as such, instead of being formed from iron as described by Hill.

The procedure finally developed is satisfactory as regards reproducibility of results, recovery of added amounts of pure lipide, simplicity, sensitivity to small amounts of lipide, and insensitivity to other variables. Some of the preliminary studies are as follows:

Tests on Fatty Acid Esters

Portions of tristearin, ethyl stearate, tripalmitin, trimyristin (Eastman), and methyl oleate and methyl linoleate (Hormel Foundation), were weighed on an analytical balance, dissolved in absolute ether and/or absolute ethyl alcohol, and diluted in a water bath at constant temperature (25°C.) so that 1 ml. of each solution contained exactly 0.01 milliequivalents (mE.), respectively, of the ester. The equivalent weights were calculated by dividing the molecular weight by the number of carboxyl groups in the molecule, *i.e.*, the equivalent weight of tristearin equals the molecular weight divided by 3.

Known amounts of the esters were added to Erlenmeyer flasks, and the color was developed by adding the reagents as described later under procedure. Spectrophotometric analyses made in the usual way with a Coleman Junior Spectrophotometer showed that the color followed Beer's law in the range tested (100-10% transmittance). Curves for all the fatty acid esters tested fell within a narrow range. The finding that there are small differences in the slope of the curves for individual fatty acids is in agreement with the findings of Feigl,

Anger and Frehden (4), who studied the minimum amount of a given ester required to produce a detectible color. When free palmitic acid was similarly tested, no color developed.

ANALYSIS OF BLOOD SERUM

Amounts of between 1 and 14 ml. of alcohol-ether extract of blood serum, prepared according to the method of Bloor (7), were measured into flasks, evaporated to dryness and redissolved in absolute ether. Color was developed and measured as described above. When the optical density was plotted against the number of ml. of extract used, a straight line resulted. When serums of different patients were used, the curve was always a straight line, but the slope varied. When portions of the same alcohol-ether extract were tested at intervals during several days, the curve was the same.

In another experiment, 1 cc. of a concentrated alcohol-ether extract of blood serum was added to each of a series of flasks, except a control. In addition, known amounts of ethyl stearate were added to all but two. The color was developed, and the results were plotted in the usual way. The flasks with only the serum extract added, yielded %T values which were plotted at 0 mE., the flask with 0.001 mE. of ethyl stearate added yielded a %T value that was plotted at 0.001 mE., and so on. A straight line resulted which had the same slope as the curve obtained with pure ethyl stearate. This indicated that the presence of materials extracted from blood serum with alcohol and ether did not interfere with the color developed from ethyl stearate. Also, when pure ethyl stearate is added to a serum extract, a quantitative recovery is possible.

Tests on Contaminating Substances

Alcohol-ether extracts of blood serum contain a number of substances that may interfere with lipide determinations (8,9,10,11). These include urea, amino acids, cholesterol, and sugar. To determine whether these substances interfere appreciably with the color development, glucose, cholesterol, urea, and glycine, were added to a known amount of ethyl stearate. The amounts of these substances normally present in 3/25 ml. of serum (the actual amount taken for analysis) was calculated. This amount, and double this amount, of each substance dissolved in alcohol were added to individual flasks containing 0.003 mE. of ethyl stearate. The color was developed and found to be the same in the flasks with the added substances as in the ones with only the ethyl stearate.

The effect of other non-lipide substances (19) that might influence the determinations was tested. Even if these substances were quantitatively extracted with the lipides, their concentration in the alcohol-ether extracts of serum would be small in comparison to the amount

of lipide present. The amounts of penicillin G, sulfanilamide, sodium salicylate, and sodium glycerophosphate present in 3/25 ml. of serum were calculated according to the following conditions and added to individual flasks containing 0.002 mE. of ethyl stearate. Serum levels of 4 units/ml. for penicillin (less than 0.000007 mE.), 20 mg.-% for sulfanilamide, and 400 γ (0.0025 mE.)/ml. for sodium salicylate, were assumed in making the calculations. The amount of phosphorus taken was double the amount normally present as lipide phosphorus. The color was developed and found to be the same in the flasks with the added substances as in the ones with only the ethyl stearate, except for the flask containing the sodium salicylate. In this flask, the per cent transmittance was 1.5 less than in the others. Thus, by comparison on the calibration graph, the lipide determination would be 3-4% too high if the sodium salicylate serum level is 400 γ /ml. [equivalent to a dose of 10 g. per day (20)], providing that salicylate is quantitatively extracted from serum with alcohol and ether.

Removal of Alcohol, Ether, and Water from the Blood Serum Extract

Since water is known to interfere with the formation of the hydroxamic acids, this was removed from the sample (3 ml.) by evaporation to dryness on a water bath. The most satisfactory temperature was found by evaporation to dryness of samples of the same extract at room temperature, at 60°C., and at 85°C. Values obtained from the samples evaporated at room temperature and at 60°C. were the same, and those from the samples evaporated at 85°C. were somewhat less, indicating that some lipide had been lost or destroyed by this temperature.

Since the time required for evaporation at 60°C. is much less than that for room temperature, the 60°C. temperature was chosen. Another factor affecting the time of evaporation is the ratio of alcohol and ether used for the extraction of the blood serum. When alcohol and ether are in the 3:1 ratio, about 2 hr. are required for the evaporation to dryness of 3 ml. of extract. With a 1:1 ratio, as described by Boyd (12), the time required for evaporation is about 1 hr. at 60°C. Gentle suction to hasten evaporation caused considerable loss of measurable lipide.

PREPARATION OF REAGENTS

Sodium Hydroxide. A 2.5% solution of NaOH is prepared in 95% ethyl alcohol, and saturated with sodium carbonate. This will last for at least one week if stoppered and stored in a refrigerator.

Hydroxylamine Hydrochloride. A 2.5% solution is prepared in 95% ethyl alcohol. This will also suffice for one week if stoppered and stored in a refrigerator. Baker's C.P. reagent was used.

Stock Iron Reagent. Two g. of white hydrated ferric perchlorate (obtained from The G. Frederick Smith Chemical Co., Columbus, Ohio) are weighed into a beaker and dissolved in 10 ml. of distilled water measured from a pipette, then 20 ml. of 71% HClO_4 are added. This stock solution is stored in a glass-stoppered flask and will keep for at least one week. It should stand about 1 hr. after preparation before being used. Half of the above quantities of ferric perchlorate, water, and perchloric acid may be prepared if only a few tests are to be made.

Since ferric perchlorate crystals are hygroscopic, accurate weighing is difficult. To test the effect of small variations in the amount of this reagent, 2 stock solutions were prepared. Stock solution A contained 0.8 g. and solution B contained 1.2 g. of ferric perchlorate. Both solutions contained 5 ml. of water and 10 ml. of perchloric acid. Separate curves were then prepared using each stock solution on portions of the same alcohol-ether serum extract. The curves resulting from use of the two stock solutions were the same.

Dilute Iron Solution. Five ml. of the stock iron reagent are pipetted into 100 ml. of 95% ethyl alcohol. This solution is prepared about 5 min. before use.

Ether. Anhydrous reagent grade ethyl ether (Merck or Mallinckrodt) was obtained in 1 lb. containers and stored in a refrigerator. Ether that has been opened and stored at room temperature becomes unsatisfactory. Anesthesia grade ether is also unsatisfactory.

PROCEDURE

Blood serum is extracted by adding 1 ml. of fresh serum to 15–20 ml. of alcohol ether mixture (3 parts of 95% alcohol to 1 part of ether) in a 25 ml. volumetric flask. The mixture is heated to boiling on a water bath, cooled, diluted to volume with the alcohol-ether mixture, and filtered.

Three ml. of the filtered extract are accurately pipetted into a 250 ml. wide mouthed Erlenmeyer flask and evaporated to dryness in a water bath at 60°C.

The flask is removed from the water bath and about 15 ml. of anhydrous ether are added. This is conveniently and rapidly done with a pipette or burette with a broken tip, since the exact amount of ether is not important. Then 0.3 ml. of NaOH solution and 0.3 ml. of $\text{NH}_2\text{OH} \cdot \text{HCl}$ solution are added. The solutions are mixed and evaporated just to dryness in a 60°C. water bath. The remaining ether vapor may be removed by inserting a pointed glass tube which is attached to a suction pump, otherwise the vapor will condense and flow down the side of the flask when cooled to room temperature.

For color development, 10 ml. of the dilute iron solution are added to the flask, which then is allowed to stand for 20 min. at 25°C. The colored solution is then poured

into a cuvette (19×150 mm.) and transmittance is read at a wave length of 520μ in the usual way. A reference standard is prepared at the same time in a similar manner, except that serum extract is not added. A model 6A Coleman Junior Spectrophotometer was used.

Calibration Graph

Because tripalmitin closely represented colorimetrically the various fatty acids tested, and because of the ease of weighing, it was chosen as most suitable for constructing the calibration graph.

One milliequivalent (mE) of tripalmitin (807/3 — 269 mg.) is dissolved in ether in a 100 ml. volumetric flask and diluted to the mark at a known temperature. One ml. of this stock standard solution at a given temperature will contain 0.01 mE of tripalmitin.

The working standard is prepared by transferring 10 ml. of the stock standard solution to another 100 ml. volumetric flask and diluting it to the mark with alcohol and ether (1:1) at a given temperature. It then contains 0.001 mE/ml. In pipetting solutions containing ether, more satisfactory results were obtained when a pipette not calibrated to the tip was used, and when the dry pipette was rinsed once or twice before use with the particular solution.

To 250 ml. wide mouth Erlenmeyer flasks are added 2, 4, and 6 ml. of the working standard solution which contain then 0.002, 0.004, and 0.006 mE of tripalmitin, respectively. These are evaporated to dryness at 60°C ., and the hydroxamic acid is formed and the color developed as described above. A reference standard is prepared at the same time in a similar manner, except that the tripalmitin is not added.

The results are plotted on semilogarithmic graph paper with percentage transmittance plotted on the logarithmic scale against mE on the arithmetic scale. The curve may be calibrated to give directly mE/l. of serum.

Methyl oleate (Hormel Foundation, Austin, Minn.) obtained in 1 g. quantities was also found satisfactory for preparation of the calibration graph. Calibration curves could be accurately reproduced from all of the samples tested. One mE of methyl oleate is 296.48 mg. or 0.338 ml. at 18°C .

Sample Calculation

If the spectrophotometer reading is 57%T, the calibration graph used may indicate that this is equivalent to 0.00155 mE of fatty acid. Since 1 ml. of serum was diluted to 25 ml., and 3 ml. of the extract analyzed, 1 ml. of serum contains $25/3 \times 0.00155$ or 0.01292 mE of fatty acid, and 1 l. would contain 12.9 mE of esterified fatty acid.

DISCUSSION

Since there is a small difference in the depth of color produced by equivalent amounts of the different fatty acids, the type of fatty acids occurring in blood should be considered. The statement of Peters and Van Slyke (13) that palmitic, stearic, palmitoleic, and oleic acids make

up the major portions of all mammalian fatty acids is well substantiated in tables published by Hilditch (14), which include detailed analyses of a number of mammalian lipides. A comparison of analysis of human depot fat by Cramer and Brown (15), and human milk fat by Baldwin and Longenecker (16), and studies on the iodine number of various fractions of blood lipides by Bloor, Blake and Bullen (17),

TABLE I
Analyses of Human Sera Selected at Random

Serum number	Esterified serum fatty acids, mE/l.
1	11.3
	11.3
	11.3
	11.5
2	12.5
	12.7
3	13.4
	13.6
4	11.4
	11.5
5	11.0
	11.5
6	12.2
	11.7
7	10.5
	10.3
8	7.3
	7.5
9	7.0
	7.4
	7.5
10	24.0
	24.5
	24.2
	25.0
11	8.9
	8.8
	9.0
	9.1
	8.9

further support this view and also suggest that the proportion of stearic acid is rather low in comparison to the proportion of palmitic and oleic acids. Because curves for palmitic, oleic, linoleic, and myristic acids are, for the purpose of analysis, nearly superimposed, palmitic acid was chosen as the best for construction of the calibration graph. However, the difference found for the slopes of the various fatty acids is such that maximum error from this source would be of the magnitude of 4%.

Another possible source of error is the splashing of water droplets into the anhydrous ether on the water bath. This is avoided easily by placing the flasks in the water bath gently. Water vapor is apparently displaced by the heavier ether vapor.

The observation of Hill that the transmittance of the colored iron complex is relatively stable about 20 min. after it is formed was confirmed. The choice of wave length was also confirmed by preparing a spectral-transmittance curve of the colored complex.

The 71% perchloric acid, when hot and concentrated, is a powerful oxidizing agent. However, when cold or dilute it is not strong enough even to liberate iodine from dissolved iodides (18).

In making extractions for routine clinical analyses, an accurately calibrated 25 ml. glass stoppered graduate may be substituted for the 25 ml. volumetric flask. Graduates are more easily cleansed of protein precipitates than are the narrow-necked volumetric flasks.

The fasting serum fatty acid levels of human adults selected at random fell in the same range (7.3-36.9 mE/l.) that Peters and Van Slyke (13) report as normal (Table I).

SUMMARY

A method is described for the colorimetric determination of the total serum esterified fatty acids based on the conversion of the fatty acid esters into hydroxamic acids and the subsequent development of colored ferric salts. Details of the procedure and sources of error are discussed.

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The Respiration of Nitrogen-Deficient Bacteria

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INTRODUCTION

In a previous paper (1) we examined the relations between the nitrogen content of the bacterial cell and the activity of some of its enzymes. We noticed that the activity of an adaptive enzyme (saccharase) suddenly decreases as soon as the N-content of the cell diminishes. On the other hand, the activity of the constitutive enzyme catalase remains fairly unchanged, and decreases only slightly in very low N-bacteria.

It is the purpose of the present paper to enlarge the former observations by a study of the relation between the respiration of bacteria and their N-content; the respiratory enzyme complex is, indeed, one of the most important enzyme systems, as it is the energy liberator for the cell.

PROCEDURE

The organism used was *Escherichia coli* (strain K 3). The cultures and bacterial suspensions were prepared by the methods of our previous paper. For details see Table I. The centrifuged bacterial mass was made "glycogen-free" according to Karström (2), harvested by renewed centrifugation, and suspended in doubly distilled sterile water. The O₂ uptake of the resting bacteria was measured immediately by the Warburg technique at pH 7 in phosphate buffer during 1 hr. with: glucose 1-2%; saccharose 1-2%; fructose 1-2%; lactose 3%; glucose 1% + fructose 1%. The oxygen uptake is expressed as Q_{O₂} (N). Estimations of N (a measure of the protein content), dry weight and saccharase, are made as in our previous paper. Saccharase activity is expressed as Lf N. Lactase was determined by the same method as saccharase: in a 24-hr. old autolyzate at pH 7 under toluene, at 37°C. on 5% lactose by using the Bertrand method. Because of the reducing properties of lactose itself, calculation of the lactase activity constant differ from the classical one for saccharase. The amount of lactose hydrolyzed is read from a graph, obtained by means of the Bertrand tables, in which the increasing reducing power is plotted against the decreasing amount of unhydrolyzed lactose. Control experiments fully justified this procedure. The lactase activity constant was calculated/g. N, with a formula anal-

ogous to that of v. Euler for saccharase, and expressed as $Lf\ N = \frac{k \cdot g. \text{ sugar}}{g. \text{ N}}$. Sterile and aseptic conditions were maintained during the manipulations.

RESULTS

Table I and Fig. 1 illustrate some of our typical experiments. The respiration was measured on the substrates of the adaptive enzymes, saccharose and lactose, and on their hydrolytic products. It must be stressed that the N percentage from the present series of experiments is not directly comparable with those of the previous paper, because this time the bacteria were made glycogen free.

In experiments on bacterial respiration it is very difficult to remove completely the endorespiration (without substrate), even by prolonged autofermentation. In our experiments the endorespiration decreased in the same proportion as the substrate respiration (Table II). Thus, whether both respirations are competitive or additional, the "blank" does not affect the outlook of the substrate respiration curve. The former is about 15-25% of the latter.

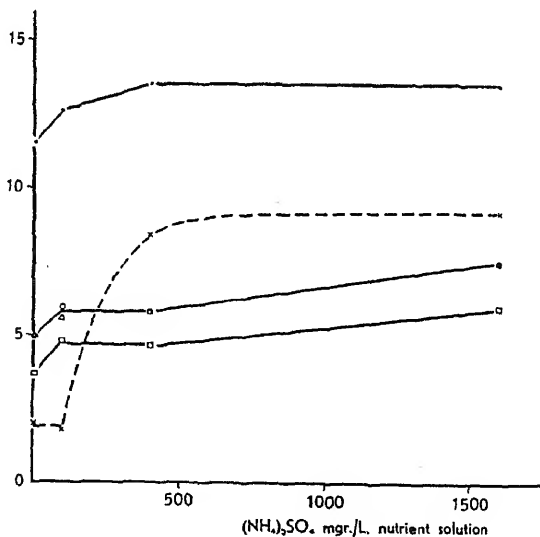


FIG. 1. Saccharase activity and oxygen uptake on different substrates with varying N-content of cells of *E. coli* (Exp. 11). ●——● N % of dry matter. X---X $Lf\ N \times 20$. ○——○ $Q_{O_2} (N) \cdot 10^{-2}$ on saccharose 2%. △——△ $Q_{O_2} (N) \cdot 10^{-2}$ on glucose 2%. □——□ $Q_{O_2} (N) \cdot 10^{-2}$ on fructose 2%.

TABLE I

Each experiment comprised 4 or 5 flasks with 3 l. nutrient solution per flask. Duration of culture: 40 hr. Temp.: 37°C. The bacteria in Exps. 11 and 15 are cultured on saccharose, those in Exp. 19 on lactose.

(NH ₄) ₂ SO ₄ mg./l. nutrient sol.	Yield of mg. dry bact.	N-content of dry bact.	If N	QO ₂ (N) on			
				Sacch. 2%	Gluc. 2%	Lact. 3%	Gluc. 1% + fructose 1%
Exp. 15		<i>per cent</i>					
6	46.4	11.3	0.088	-519	-385		-455
20	61.4	11.6	0.096	-564	—		-470
100	339	12.1	0.112	-481	-492		-435
400	367	13.2	0.424	-450	-510		-487
1600	540	13.4	0.440	-565	-546		-510
Exp. 19			If N				
6	53.9	4.46	0.110		-355	-443	
20	118	4.89	0.076		-625	-752	
100	439	6.51	0.320		-815	-947	
400	914	7.97	0.344		-918	-918	
1600	810	7.83	0.646		-794	-814	

TABLE II

Showing that the Endorespiration is Proportional to the Substrate Respiration with Varying N-Content of the Culture Medium

(NH ₄) ₂ SO ₄ mg./l. nutrient sol.	QO ₂ (N)			
	Exp. 40		Exp. 41	
	Glucose. 1%	Endoresp.	Glucose. 1%	Endoresp.
1600	-612	-73	-366	-79
50	-548	-60		
6			-196	-55

Some *coli* strains oxidise glucose and the other carbohydrates with CO₂ and H₂ formation. The aerobic H₂ evolution with our strain is very small or zero; indeed, we noticed that, first of all, no hydrogen evolution could be measured with colloidal palladium (3, 4) in the Warburg vessel (the activity of the Pd preparation was controlled under the

same conditions with H_2 from fermentation on $HCOOH$ and from Mg and H_2SO_4); secondly, respirations measured on succinic and ascorbic acid (not recorded in this paper), from which a formation of H_2 can hardly be expected and could not be demonstrated either, result in exactly the same curves as shown in Fig. 1. The recorded values for the oxygen consumption will thus approximate the real ones very closely.

DISCUSSION

Our experiments show that there is only a small decrease of oxygen uptake by the cells when the N-content is diminishing. Only with bacteria (Exp. 19) containing very little nitrogen, O_2 uptake is noticeably lower. The decrease in the respiration is mostly 10–30%, in the extreme cases 45–55%.

On the other hand the activity of the adaptive hydrolases, saccharase and lactase, falls down abruptly as soon as the nitrogen content diminishes, and stabilizes at about 10–20% of its original value.

The difference in deportment of the adaptive enzymes and of the respiration is striking in all the experiments, especially at the beginning of N-deficiency. Thus, in the instance of protein deficiency, the respiratory enzyme system is not so much damaged as are the adaptive enzymes, which are less immediately concerned with the maintenance of life. Virtanen has discussed this problem from many viewpoints (5). In connection with the above results it is interesting to refer to the experiments of Holter and Zeuthen (6), from which analogous conclusions can be drawn concerning the constancy of the respiratory system. From investigations on the endorespiration of the starving amoeba *Chaos chaos* L., they stated that "the overall rate of metabolism is preserved": the O_2 uptake/hr./ γ reduced weight (surplus weight of an object which is suspended in water) remains almost constant during the whole starvation period until death, although there is a great loss of "heavy substrates": very probably for the most part proteins.

In some experiments even an initial slight increase of the oxygen consumption has been noticed (e.g., Exp. 19) which is hard to explain. We assume that this increase is only apparent, and is due to the fact that the nitrogen constituents of the cell, which cannot be determined with our Kjeldahl method (destruction time: 1 hr.) as the purines and pyrimidines (hemedervatives, coenzymes, etc.), diminish more slowly in N-deficiency than the proteins. This point is under investigation.

We draw attention to the fact that, in low-N bacteria, the lactase and saccharase activity is very small, so that one may wonder whether the amount of disaccharide split is sufficient to supply hexoses for respiration, or if there is any direct way of metabolism, such as has been described by Doudoroff *et al.* (7) and Myrbäck and Vasseur (8). Measurements of the respiration on saccharose 2% and on a mixture of glucose 1% plus fructose 1% show that the rate of oxygen uptake has in all cases diminished in exactly the same proportion, even in bacteria with the lowest hydrolase activity. Thus, even bacteria with the lowest hydrolytic power are still able to supply enough hexoses from the disaccharides, so that a direct way of metabolism of this substances must not be considered in *E. coli*.

ACKNOWLEDGMENTS

We are indebted to Prof. A. I. Virtanen, the preliminary experiments being performed in his Laboratory of the Foundation for Chemical Research, Helsinki, and to Prof. L. Massart for his interest and encouragement in the problem.

SUMMARY

E. coli is grown on media containing different N amounts, ranging from 6 to 1600 mg. $(\text{NH}_4)_2\text{SO}_4/\text{l.}$ The nitrogen content of the bacteria diminishes with decreasing N in the medium. The respiration per mg. N of bacteria diminishes about 10–30%, in utmost cases about 50%. The activities of the adaptive hydrolases, saccharase and lactase, decrease to 80–90% of their original value. Thus, in the instance of nitrogen deficiency the respiratory enzyme system is not so greatly damaged as are the adaptive enzymes.

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The Effect of Autoclaving with Dextrose¹ on the Nutritive Value of Casein

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INTRODUCTION

A number of studies on the effect of heat treating proteins by autoclaving and dry heating have been reported in recent years dealing chiefly with the effect on their growth promoting qualities. However, few attempts have been made to determine the effect of heat treatment of proteins in the presence of reducing sugars on the growth promoting qualities of the proteins or protein derivatives. Stevens and McGinnis (1) have shown that lysine autoclaved in the presence of cerelose was either destroyed or rendered unavailable for chick growth. Hill and Patton (2) found that the slight discoloration occurring during autoclaving of the media for the microbiological assay of L-tryptophan was caused by interaction with glucose, resulting in decreased growth of *Streptococcus faecalis* R. Later work by these investigators (3) showed this decrease in growth was due not to formation of growth inhibitors as products of the browning (Maillard) reaction but to actual destruction of part of the tryptophan. This destruction, however, was not limited to tryptophan, as both L-lysine and DL-methionine, upon heating in the presence of glucose, underwent similar destruction. In this paper, we wish to report the effect of heat treatment in the presence of a reducing sugar on the growth promoting value of casein.

EXPERIMENTAL

Plain casein (General Biochemicals, Inc.) was used in these studies. To this crude protein was added a dextrose solution so prepared as to contribute 0.5 ml. distilled water/g. of crude casein and 1 g. dextrose (anhydrous-Merck) for every g. of protein

¹ During preparation of this manuscript, a paper appeared, by Patton, Hill and Foreman (7), which demonstrated a decrease in the lysine, arginine, and tryptophan content of casein heated with dextrose.

(N \times 6.38) present in the casein. This mixture of casein and dextrose was autoclaved for 2 hr. at 250°F., the resulting material being dark brown and quite tough and rubbery, and containing no detectable free amino groups (Table I). To determine the effect of dextrose at room temperature, a similar mixture of casein and dextrose as above was air-dried at room temperature.

As controls, to show the effects of autoclaving in the absence of dextrose, the crude casein and casein to which was added the same ratio of distilled water as contributed by the dextrose solution were autoclaved for 2 hr. at 250°F. The casein autoclaved "as is" formed a hard, brittle, buff-colored mass, whereas the casein autoclaved with water formed a tough orange-colored gel.

TABLE I
Calculation of Biological Value

Diet	Free amino N (Van Slyke)	Average initial weight	Average final weight	Average total weight change	Average weight food eaten	Protein in diets	Average total protein intake	Average gain or loss/g. protein intake	Biological value (Casein 100)
		g.	g.	g.	g.	per cent	g.		
A. Casein autoclaved with dextrose	0.00	55	45	-12 \pm 1	123	10.8	13	- .87 \pm .07	—
B. Casein and dextrose dried at room temp.	0.21	54	108	54 \pm 3	216	10.7	23	2.33 \pm .09	100
C. Casein autoclaved with water	0.34	56	98	42 \pm 4	214	10.3	22	1.85 \pm .16	79
D. Autoclaved casein	0.99	57	102	45 \pm 2	239	10.4	25	1.82 \pm .27	78
E. Untreated casein	0.24	55	112	57 \pm 2	227	10.8	24	2.34 \pm .08	100

Isocaloric diets A, B, C, D, and E were prepared, containing, in addition to their respective casein components at 10% protein level, fat as Mazola Oil (Corn Products Refining Company) to 6%, Salt Mixture No. 2 (U.S.P. XII Wyeth & Company) 4%, and sufficient starch to make the diets isocaloric. To each kg. of diet was added a vitamin supplement of choline chloride 1.0 g., α -tocopherol acetate 0.10 g., pyridoxine HCl 0.002 g., calcium pantothenate 0.005 g., riboflavin 0.037 g., and flour enrichment (Type 5-N-Richment A, Novadel-Agenc Corp.) 0.9 g. Vitamins A and D were added as a daily supplement to each animal's food.

Five groups of weanling albino rats (Carworth Farms) were used for the feeding test, one group for each diet. Each group consisted of 5 male and 5 female weanling animals 28-29 days of age. The animals were so placed on test that the average weights of the animals of each sex were comparable within the groups.

The feeding test was carried out for 28 days, during which the animals were weighed at 4 day intervals. Daily observations were made for abnormalities in appearance and physiological activities. At the conclusion of the assay period, the animals were sacrificed and autopsied for gross pathology.

All animals were placed on test on the same date and were fed *ad libitum* until it was evident that the animals receiving the casein heated with dextrose (Diet A) were

eating the least. The daily average food intake of male and female animals of this group controlled the daily intake of the respective sexes of the other 4 groups until it was noticeable that Diet A animals were rapidly losing weight and would jeopardize the paired feeding technique. At this time, group A was fed *ad libitum* while groups B, C, D, and E remained on paired feeding, their food intake being controlled by the group other than A that consumed the least food.

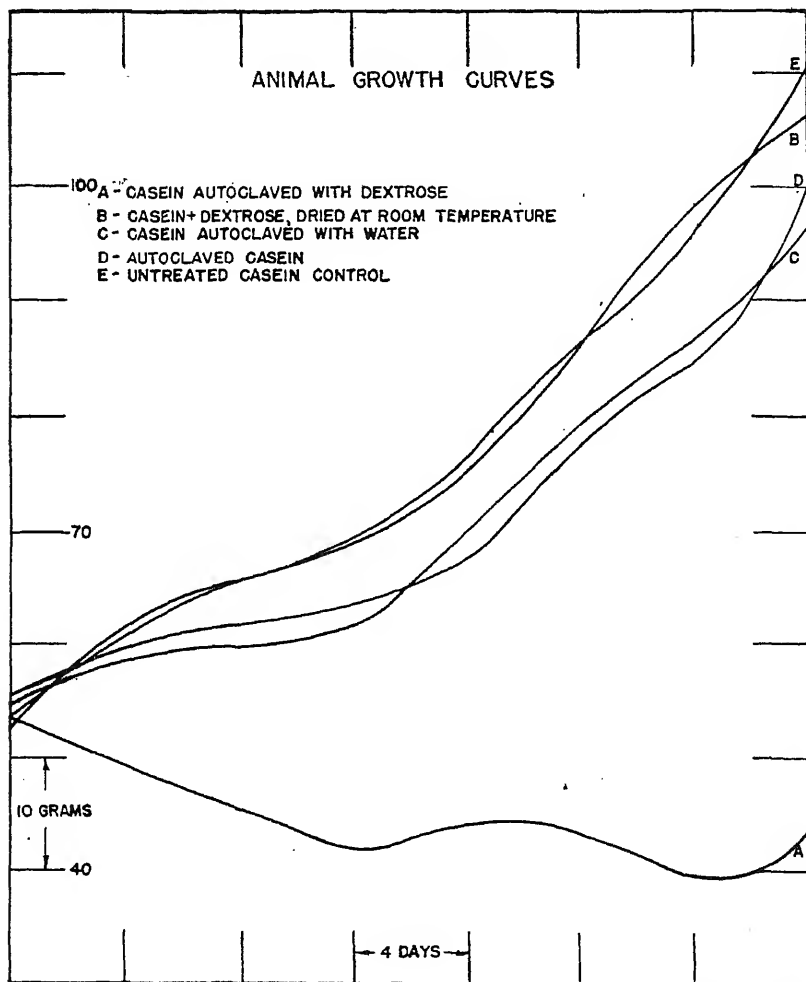


FIG. 1. Animal growth curves showing the impairment in growth on Diet A containing the autoclaved casein-dextrose.

RESULTS AND DISCUSSION

During the 28 day test period, animals on Diet A, receiving casein autoclaved with dextrose, rapidly lost weight (Fig. 1), 2 animals succumbing on the 20th day. The animals receiving casein and dextrose dried at room temperature (Diet B), casein autoclaved with water (Diet C), autoclaved casein (Diet D), and the casein control (Diet E), grew favorably, the animals receiving Diet B growing as favorably as the control animals. In accordance with results obtained by previous investigators (4,5) on the effect of heat-treating casein, autoclaved casein and casein autoclaved with water had a lower biological value than the unheated protein (Table I). However, when the casein was autoclaved in the presence of dextrose, its biological value was lowered to a point where it would no longer promote tissue synthesis. Drying with dextrose at room temperature had no apparent effect on the growth promoting qualities of the casein, the biological value of this material (B) being comparable to the untreated casein control.

During the test, abnormalities in appearance and physiological activities were prevalent in animals fed casein autoclaved with dextrose (Diet A). Attendant with a marked loss in weight in these animals, was the development of a pasty-white appearance of ears and feet, a reddish appearance of the head, bedraggled appearance of fur and, as the test progressed, a slightly humped appearance, encrustation of the eyes, and a bloody whitish discharge from the nostrils. In the early stages of the test, the animals were slightly nervous but, as the test progressed, they became very sluggish. Two animals of this group, both exhibiting the above typical symptoms of unbalanced and inadequate amino acid intake, died on the 20th day of test. Throughout the test, the feces of animals receiving Diet A were black and sticky. Animals fed diets B, C, D, and E exhibited no typical symptoms of unbalanced and inadequate amino acid intake.

Autopsy for gross pathology showed abnormalities prevalent in animals receiving casein autoclaved with dextrose. Each animal of this group had a small light-colored liver, discolored kidneys and spleen, and slightly haemorrhagic intestines. No significant prevalence of any gross pathological condition was noted in any of the other groups.

The destruction of the growth promoting qualities of casein by autoclaving with dextrose may be in part explained by reference to data of Table I, in which it will be noted that the free amino nitrogen

(Van Slyke) (6) of the casein protein was reduced to zero by heat treatment in the presence of dextrose. Presumably the aldehyde group of the reducing sugar reacted with free amino groups of the amino acids of the proteins through typical mechanisms of the Maillard (browning) reaction. Certain essential amino acids so reacted may be rendered nutritionally unavailable to the animal system, thereby disrupting the essential amino acid balance of the casein, resulting in a drastic decrease in its growth-promoting qualities. It is suspected that the unavailability of any amino acids so reacted may be due to the formation of an enzyme- and acid-resistant linkage.

The reduction in the biological value of the casein heated with water alone may be due to the reaction of some of the essential amino acids with reducing sugars inherent in the casein protein complex.

Deamination and decarboxylation are not excluded as factors in the reduction of the biological value of the heated casein.

ACKNOWLEDGMENT

The authors appreciate the technical assistance of K. W. Schwartz. The analytical work was carried out by the Analytical Section of Central Laboratories.

SUMMARY

Casein autoclaved for a 2 hr. period at 250°F. in the presence of a large amount of dextrose (1 g./g. of protein) was nutritionally impaired to such a degree that it would not support the growth of weanling albino rats. When similarly treated in the absence of dextrose, its growth-promoting quality was only slightly impaired. Drying with dextrose at room temperature had no effect on the nutritive value of the protein.

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Studies on Apyrases. I. Purification of Potato Apyrase by Fractional Precipitation with Ammonium Sulfate ¹

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INTRODUCTION

Potato apyrase was first described by Kalekar (1), who made water extracts of potato, salted out inert proteins by adding ammonium sulfate to about 60% saturation and then precipitated the apyrase in the supernatant by full saturation with ammonium sulfate. By fractional precipitation with ammonium sulfate, adsorption of pigmented material on aluminum hydroxide, and final fractional precipitation with alcohol, a preparation was obtained which split 9 γ of phosphorus / γ of protein in the standard test. Kalekar found that potato apyrase is activated by calcium ions and that the enzyme splits off two groups of phosphate from ATP.

Meyerhof (2) stated that Kalekar's method of preparation of apyrase failed in his hands, probably owing to differences in the extraction procedure. A modified method was suggested wherein a large part of the inert proteins was precipitated by 0.4 saturation of the extract with $(\text{NH}_4)_2\text{SO}_4$. The solution was concentrated after addition of a further amount of solid $(\text{NH}_4)_2\text{SO}_4$, until it reached full saturation. The precipitate was dissolved in water, dialyzed for 2 hr. and centrifuged. The final solutions contained 5–12 mg. of protein/cc., and 0.1 cc. of such a solution split 200–300 γ of hydrolyzable phosphorus from ATP in 5 min., at 30°C., at pH 6 and in the presence of glutathione. These figures would correspond to less than 1 γ of phosphorus split / γ of protein in the standard test. When added directly to hexose diphosphate the enzyme preparation brought about a very slow dephosphorylation, because of contamination with an ordinary phosphatase.

¹ A preliminary report has been published. [KRISHNAN, P. S., *Arch. Biochem.* **16**, 474 (1948)].

* Government of India scholar.

In 1947 Kalckar (3) proposed another method of preparation of apyrase for use in differential spectrophotometric determination of adenine compounds. Potatoes were extracted with 3 volumes of 0.1 *M* $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$, pH 8.5, the extract saturated with $(\text{NH}_4)_2\text{SO}_4$ and the precipitate redissolved in 0.1 *M* $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$, pH 8.5. The solution was treated with aluminum hydroxide to remove pigmented material and inert material precipitated by the addition of 0.5 volume of saturated $(\text{NH}_4)_2\text{SO}_4$. The supernatant was used directly as the source of the enzyme, or else the protein was precipitated by 0.8 saturation with $(\text{NH}_4)_2\text{SO}_4$ and redissolved. The pH optimum was stated to be 6.8 and calcium ions were added to insure full activity.

The author's interest in the problem arose from the fact that, when the purification of the enzyme by the fractional ammonium sulfate precipitation methods of Kalckar and of Meyerhof was attempted, many of their findings could not be confirmed. Also, many of the final preparations split off practically all 3 phosphate groups from ATP, when sufficient time (30–60 min.) was allowed for incubation of the enzyme with substrate. In view of the important role that apyrase plays in many of the metabolic processes, it was decided to study in detail methods of purification of the enzyme from potato. During the course of these investigations it has become evident that different samples of potatoes show wide divergencies in activity. Although the author has not experimented thus far with potatoes harvested and stored under standard conditions—the supply used in the experiments was drawn at random from local market stores—the data tend to indicate that new potatoes obtained in May and June have a much higher activity than stored potatoes available in October and November.

EXPERIMENTAL

Standard Test

The test system was made up as follows:

- 1.0 cc. of enzyme solution
- 0.5 cc. of *M*/10 succinate buffer, pH 6.5
- 0.2 cc. of calcium chloride, containing 1 mg.
- 0.3 cc. of ATP solution, containing 100–150 γ total phosphorus
- 2.0 cc. Total volume

The mixture was incubated at 30°C. for 30 min., the reaction stopped by the addition of 1–2 cc. of 20% trichloroacetic acid, the precipitated proteins removed by centrifugation, washed once with 2% trichloroacetic acid and the inorganic phosphorus in the combined supernatants estimated by the method of Sumner (4). A unit of enzyme was defined as that amount which in the standard test outlined above liberated 1 γ of inorganic phosphorus.

Activity of Whole Potato

The activity of whole potato was estimated on the assumption that the entire activity passed into the aqueous extract when the sliced material was ground in a Waring Blendor for about 3 min. For reasons explained in a later section, $M/100$ KCN was usually employed for the extractions, but identical results were obtained also when water alone or salt solution was used as the extractant.

About 150–200 g. of potato were taken for each experiment. The samples were cut into slices and ground in a Waring Blendor for 3 min., using about 1.2 volumes of ice cold neutralized $M/100$ cyanide solution. The material was pressed through cheese cloth and the extract centrifuged in the cold for 30–45 min. at a speed of 1600 r.p.m. An opalescent solution was obtained, from which an aliquot was pipetted out and the activity estimated after suitable dilution, usually 50–200 times.

The activities/g. of fresh potato, as determined by the method described above, are presented in Table I. When the analyses were carried out in October and November of

TABLE I
Apyrase Activity of Potato

	Units/g. fresh weight
October, 1947	6139
	2702
	9422
	10800
	2591
	1955
November, 1947	4392
	2226
	3459
	2951
	3190
	4905
New potatoes, red skin, March, 1948	3920
	11570
	13560
	12230
	11760
New potatoes, red skin, May, 1948	10280
	9348
	8774
	8752
New potatoes, white skin, May, 1948	7711
	6921
	7582

1947, no attention was paid to the type of potato; probably all the samples had been stored for some time. However, during the analyses carried out in the spring of 1948, a record was kept as to whether the potatoes were of the white or red skin variety, and also whether they were new or stored.

The figures reported in Table I reveal the fact that there exists a wide divergency in the activity of different samples of potato.

Purification of Apyrase by Precipitation with Ammonium Sulfate

The data presented in the following sections on the purification of apyrase of potato relate to experiments in which $(\text{NH}_4)_2\text{SO}_4$ was the only reagent used for effecting the purification. Kalckar, and apparently also Meyerhof, used water for extraction purposes; the former reports that his final solutions were highly pigmented. In the present investigation this disadvantage has been circumvented by the use of *M*/100 neutralized KCN for extraction purposes.

A. Quantitative Study of the Fractional Precipitation Methods of Meyerhof and of Kalckar

Several preliminary quantitative distribution studies were carried out on potato extract when fractionated by the techniques of Meyerhof and of Kalckar. Five hundred to 750 g. of potato were used in each experiment. These experiments were conducted in the fall of 1947, when a record was not kept of the type of potato used. Potato was cut into bits, ground in a Waring Blendor with 1-1.2 volumes of ice cold cyanide solution (or ice cold water in some experiments) for 3 min., the material pressed through cheese cloth and the extract centrifuged in the cold at moderate speed (1600 r.p.m.) to remove starch grains and any cell debris. The activity was estimated on an aliquot and the rest of the extract was brought to 0.4 saturation by the addition of 30 g. of $(\text{NH}_4)_2\text{SO}_4$ /100 cc. of solution, and the material filtered through Whatman filter paper No. 1. The filtrate was then taken to full saturation by the addition of more solid ammonium sulfate and the precipitate collected on filter paper. The two precipitates were separately taken up in water and the activity determinations carried out after suitable dilution, and expressed as per cent of the total activity present in the initial extract. In another series of experiments the initial extract was brought to 0.6 saturation by the addition of 45 g. of $(\text{NH}_4)_2\text{SO}_4$ /100 cc., the precipitate collected on filter paper and the filtrate taken to full saturation with $(\text{NH}_4)_2\text{SO}_4$ and filtered. The two solid fractions were taken up in water and assayed for activity separately.

The data are presented in Table II.

The figures in Table II show clearly that, under the conditions of extraction and precipitation employed in these experiments, 0.6 saturation with ammonium sulfate brings down practically the entire activity of the extract, instead of serving to remove inert material. When fractionation is attempted by initial 0.4 saturation with ammonium sulfate

TABLE II
*Quantitative Fractionation of Apyrase Activity of Potato Extract
 with Ammonium Sulfate*

	Sample number	Per cent of original activity	Per cent of original activity
		In precipitate on 0.4 saturation	In precipitate on full saturation of filtrate
1. Meyerhof's method	1	55.5	34.2
	2	31.5	90.0
	3	21.5	103.0
	4	52.4	51.9
	5	48.5	96.2
	6	41.3	81.8
		0.6 saturation	Full saturation of filtrate
2. Kalekar's method	7	182	2
	8	137.9	3.3
	9	122.6	traces

and subsequent full saturation of filtrate, it is found that a considerable amount of the activity is precipitated and lost along with the solid fraction formed on 0.4 saturation. It will also be noted that different samples of potatoes yielded different figures for the fractional analysis, and that, in every case, the activity recovered was greatly in excess of the total activity of the initial extract.

In the following studies on purification of the enzyme, the author has, therefore, always carried on an initial 0.6 saturation of the whole extract with ammonium sulfate, collected the precipitate, and used this material as the starting point for further purification of enzyme. The ultimate purification was effected by fractionation with ammonium sulfate, but two sets of experiments were set up, one, in the which fractionation was carried out as the last stage after 2-3 successive 0.6 saturation precipitations, discarding the solid fractions obtained at each stage on dialysis of the material against distilled water, and a second, in which the fractional precipitation with ammonium sulfate was carried out directly on the material obtained by the first 0.6 saturation stage. The purest preparations were obtained by the first method,

but there is no doubt that similar preparations can also be obtained by the second method by repetition of the fractionation procedure.

B. Large Scale Fractionation of Potato Extract

Method 1. 1337 g. of new red skinned potatoes were ground in a Waring Blender with an equal volume of $M/100$ cyanide solution and the pressed extract centrifuged in the cold. 2120 cc. of solution was obtained which had a total activity of 13,740,000 units. The extract was allowed to reach room temperature and then precipitated by the addition of $(\text{NH}_4)_2\text{SO}_4$ to 0.6 saturation (45 g./100 cc. of extract) with mechanical stirring. The material was filtered through Whatman filter paper No. 1 and allowed to drain overnight in the cold room (4°C). The next morning the precipitate was taken up in water, when a cloudy suspension was obtained, with a volume of 355 cc. The activity was estimated and found to be 15,630,000 units, that is, representing a recovery of nearly 114%. The suspension was dialyzed against distilled water in the cold room for 24 hr. with frequent changes of the outside water. (The main filtrate from 0.6 saturation was taken to full saturation with ammonium sulfate; the precipitate formed contained only 1.2% of the total activity and was, therefore, rejected.) At the end of the dialysis it was observed that a flocculent precipitate had settled out. Estimation of the activity revealed that the enzyme in the form of suspension had an activity of 1091 units/mg. dry weight. The suspension was centrifuged in the cold for 1.5 hr., the residue washed once on the centrifuge with ice cold water and the activities in the combined supernatants and in the residue separately determined, the latter after suspending the solid material in water. Of the activity present in the whole suspension, 58.8% had passed into the supernatant and 38.1% was present in the residue. Analysis of the dry weight of the two fractions indicated that the supernatant had an activity of 1613 units/mg. dry weight and the residue 849.2 units/mg. This insoluble fraction, which separates out in every case on dialysis of the initial 0.6 saturation precipitate and which will hereafter be called the *insoluble apyrase fraction*, was set aside for separate study, and further purification was carried out with the water-soluble fraction.

800 cc. of the supernatant was brought to 0.6 saturation with $(\text{NH}_4)_2\text{SO}_4$ and the precipitate filtered, dissolved in water and dialyzed as before, and centrifuged from a small amount of residue, which carried down only a small fraction of the activity. The solution had now an activity corresponding to 1914 units/g. dry weight and the net activity in the whole solution was 5,555,000 units. To test whether any activity was being lost in the main filtrate from the second 0.6 saturation precipitate, this solution was fully saturated with $(\text{NH}_4)_2\text{SO}_4$; the precipitate formed contained only 3.2% of the total activity and was, therefore, discarded.

The clear pale yellow solution (152 cc.) obtained after 2 precipitations by 0.6 saturation as described above, was now fractionally precipitated with $(\text{NH}_4)_2\text{SO}_4$. The powdered solid was slowly added with gentle mechanical stirring. When 0.1 saturation was reached with the addition of 11.3 g. of solid, there was practically no precipitate. Another equal weight of the solid was added and the fairly bulky precipitate that separated out on 0.2 saturation was filtered off. The filtrate was treated with a third lot of 11.3 g. of ammonium sulfate, the precipitate collected and the

filtrate taken successively to 0.4 and 0.6 saturation with $(\text{NH}_4)_2\text{SO}_4$. In each case the precipitate was taken up in water, activity estimated, and dialyzed against distilled water in the cold room for 24 hr. and the activity again estimated.

The results obtained in this experiment, and in another using a different sample of potato are represented in Table III, where the distribution of activity is expressed as per cent of the activity present in the solution before fractionation. These figures were calculated on the basis of the activities prior to dialysis of the various fractions; after dialysis there were losses ranging from 5 to 20% in the various fractions.

The figures given in Table III clearly show that the precipitate formed by 0.6 saturation with ammonium sulfate, after removing the 0.4 saturation precipitate, contains the purest fraction of the enzyme, the activity being as high as 66,600 and 60,800 units/mg. dry weight.

TABLE III
Fractionation of Soluble Apyrase with Ammonium Sulfate

Sample	Degree of saturation	Per cent of total activity	Activity/mg. dialyzed solution
No. 1	0.2	3.4	
	0.3	16.3	617
	0.4	18.3	1,592
	0.6	25.3	66,600
		63.3 Total	
No. 2	0.2	6.6	277
	0.4	28.5	613
	0.6	21.4	60,800
		56.5 Total	

The potatoes used in the first experiment had an activity of 56.7 units/mg. dry weight. The purified fraction of apyrase therefore represented an 1170 fold purification on the basis of dry weight of potato. In the second experiment, the potato used had an initial activity of 22.3 units/mg. dry weight. The final preparation of enzyme, therefore, represented a 2700 fold purification on a dry weight basis. The fractionation studies also indicated that the procedure was attended with considerable losses; the activity present in the purified fraction was only

20–25% of the activity in the solution prior to fractionation. On the basis of the total activity present in the initial extract from potato, the activity recovered in the final fraction represented a recovery of 7.3% in the first experiment and 15.9% in the second.

Method 2. In this method the precipitate obtained in the first stage by 0.6 saturation of potato extract with $(\text{NH}_4)_2\text{SO}_4$ was filtered, suspended in water and fractionated with $(\text{NH}_4)_2\text{SO}_4$ without prior dialysis. It should be emphasized that in these fractionations too much significance should not be attached to the degree of saturation, because at several stages the filtrations were slow and had to be carried out, sometimes overnight, in the cold room, thereby altering the solubility of $(\text{NH}_4)_2\text{SO}_4$; also, no special precautions were taken to make allowance for any volume changes accompanying precipitation and removal of the solid matter at the various stages.

Four experiments were carried out, starting with 1.5–2.0 kg. new potato each time, using red skinned potato in 2 experiments and white skinned potato in 2 others. Each experiment took over a week for completion. The precipitates obtained by fractionation were separately dialyzed against distilled water in the cold and a study was made of the distribution of activity between the solution and the residue, if any, separating during the dialysis. The results are represented in Table IV.

An examination of the data in Table IV reveals many interesting features. The figures vary from sample to sample, but, in general, the fraction separating out on 0.4 saturation, after removal of the 0.2 saturation precipitate, contains the purer enzyme. The activity of this fraction varies from 3200 to 6200 units/mg. dry weight. This fraction deposits only a small amount of solid material on dialysis and the solid fraction possesses practically the same activity as the solution on mg. dry weight basis. As contrasted with this, the fraction precipitated on 0.2 saturation with ammonium sulfate gives rise to considerable amounts of solid matter on dialysis and the solid fraction carries down a major fraction of the activity. The most active solution obtained, namely, that having an activity of 6193 units/mg. dry weight, is still just a tenth as pure as the purest fractions obtained by the first method of fractionation, but there is no doubt that further fractionation of these solutions should yield the purified product, as illustrated by the following experiment.

The soluble apyrase fractions obtained by dialyzing the 0.4 saturation precipitates against distilled water and discarding the residues in the 4 experiments outlined above, had been stored in the cold room for periods ranging from 2 to 6 weeks. They were finally combined and analysis showed that the mixed solution had an activity corresponding to 2865 units/mg., indicating that a certain amount of inactivation had taken place during the storage. The solution was brought to 0.4 saturation with $(\text{NH}_4)_2\text{SO}_4$,

TABLE IV
Fractionation of Apyrase Present in the Initial 0.6 Saturation Precipitate

Sample	Degree of saturation	Per cent of total activity	Activity/mg. dry weight of dialyzed suspension	Per cent distribution of activity in	
				Solution	Residue
White No. 1	0.2	49.0	793	42	70
	0.4	38.9	6193	71.9	12.3
	0.6	traces			
		87.9 Total			
No. 2	0.2	35.6	638	16.1	75.4
	0.4	48.3	3200	92.0	20.1
	0.6	1.6			
		85.5 Total			
Red No. 1	0.2	23.6	378	70.7	36.2
	0.4	55.8	3895	76.5	14.6
	0.6	1.0			
	0.8	nil			
		80.4 Total			
No. 2	0.2	43.3	568	35.2	55.3
	0.4	42.8	4083	49.1	42.3
	0.6	traces			
		86.1 Total			

the precipitate formed filtered, the filtrate taken to 0.6 saturation with $(\text{NH}_4)_2\text{SO}_4$ and filtered again. The two precipitates were separately dissolved in water and dialyzed against distilled water in the cold and separated from a small amount of residue by centrifugation. Analyses of the solutions showed that the 0.4 saturation fraction had an activity of 1932 units/mg., and the 0.6 saturation fraction 13,680 units/mg.; 66.2% of the initial activity had passed into the latter fraction, as against 28.7% into the former. The experiment also reveals a significant fact, which was apparent from the experiments reported under Method 1, namely, that from partially purified aqueous solutions, the apyrase activity always becomes concentrated in the protein fraction separating on 0.6 saturation, after discarding the precipitate formed on 0.4 saturation.

DISCUSSION

Fractional precipitation of potato extracts with ammonium sulfate effects a considerable purification of soluble apyrase, the activity being associated with the protein fraction precipitating between 0.4 to 0.6 saturation of partially purified solutions. An appreciable part of the total activity, amounting to 25–40%, is intimately bound with insoluble material separating during dialysis. Enough data is not available yet to decide whether the presence of apyrase activity in the insoluble fraction should be attributed to mechanical adsorption, or formation of complexes of more or less stable nature, or even partial denaturation of enzyme. In this connection, it is interesting to recall that apyrases have been known to be intimately associated with insoluble material. Kalekar (1) reported that, when solutions of potato apyrase are mixed with myosin, a considerable proportion of the former is bound to the myosin fraction and cannot be washed out subsequently by simple means. Meyerhof (2) stated that apyrase of yeast is bound to the cell structure. Steinbach and Moog (5) showed that apyrase of chick embryo tends to be associated with the granular fraction. Barth and Jaeger (6) obtained from frog eggs several protein fractions showing apyrase activity, and considered the possibility of formation of complexes between apyrase and inert proteins. Meyerhof and Geliazkova (7) showed that, in the case of brain tissue, the apyrase activity is strongly adsorbed by the structural elements. Whatever the reasons may be for the presence of apyrase activity in the insoluble fractions separating from potato extracts, the enzymic properties of the soluble and insoluble fractions seem to be practically identical, as reported in the second publication of this series (8).

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1. Different lots of potatoes contain widely different concentrations of apyrase. This may be connected with conditions of storage.
2. On fractionation with ammonium sulfate, the apyrase activity of cyanide extracts of potato passed into two distinct fractions, an insoluble fraction which separates during dialysis of the precipitated

material against distilled water, and a soluble fraction. The latter has been purified further by one or more reprecipitations and a final fractional precipitation with ammonium sulfate. A very potent preparation has been obtained, in yields of 7–15% of the initial activity. This is practically colorless, has an activity of over 60,000 units/mg. dry weight, and represents a 1000 to 2700-fold purification on the basis of initial dry weight of potato.

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Studies on Apyrases. II. Some Properties of Potato Apyrase

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INTRODUCTION

The occurrence of adenylpyrophosphatase, which splits 2 groups of phosphate from ATP was demonstrated in muscle by Lohmann (1), and in liver by Jacobsen (2) and by Barrenscheen and Lang (3). A one-step dephosphorylation of ATP was first shown by Lohmann (4), who found that well-washed lobster muscle residue splits only the terminal phosphate group of ATP. In 1939, Engelhardt and Ljubimowa (5) made the important discovery that the adenylpyrophosphatase action of muscle is intimately associated with the myosin fraction, and that, whereas crude extracts of myosin split off both the labile groups of ATP, myosin reprecipitated 3 times liberated only the terminal group. It was generally assumed that the second phosphate group of ATP was split by a different enzyme, an adenyldiphosphatase, which is water soluble.

Kalekar (6), however, contradicted the existence of an adenyldiphosphatase; he described a new enzyme, myokinase, which dismutates two ADP molecules to give a molecule each of ATP and AMP. The ATP so formed is dephosphorylated again to ADP and so the reaction goes on until all the ATP is dephosphorylated to AMP. Kalekar's theory was criticized by Szent-Györgyi and his school (7, 8), who discounted the role played by myokinase in bringing about the splitting of the second phosphate group; they postulated that the second phosphate group of ATP is split by myosin itself, but only in the presence of a system made up of myosin and a water-soluble acid-stable protein, which they called "Protein 2." Szent-Györgyi's recent finding that recrystallized myosin splits 2 phosphate groups from ATP demonstrates that no simple explanation is yet available for the mechanism of dephosphorylation of ATP by muscle tissue. Kielley and Meyerhof (9) have just reported the presence in high concentration of a new ATP-ase in muscle, which can be completely separated from

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myosin ATP-ase and which behaves antagonistically toward the latter with respect to calcium activation and pH optimum.

DuBois and Potter (10) reported that other tissues, in addition to muscle, contain an enzyme system which splits inorganic phosphorus from ATP. Tissue homogenates were used for measuring the enzymic activity and no data were reported as to how many phosphate groups were split off from ATP. Kalckar (6) investigated the adenylypyrophosphatase activity of liver extracts and also described the purification and some of the properties of adenylypyrophosphatase of potato. Both the preparations were free from myokinase; nevertheless, two molecules of phosphate were split from ATP. He, therefore, made a rough distinction between adenylypyrophosphatase of myosin preparations, which is an adenosinetriphosphatase, and adenylypyrophosphatases of liver and potato which split off phosphorus from both ATP and ADP. He considered that it is not necessary to postulate the existence of separate ATP-ase and ADP-ase; some adenosine pyrophosphatases are less specific so that they can act on ADP as well as ATP. Meyerhof (11) first suggested the name *apyrase* to describe those adenylypyrophosphatases which act on both ADP and ATP, and split off 2 phosphate groups from the latter.

This class of enzymes is to be distinguished from the *true ATP-ases* which split only the terminal phosphate group of ATP. Meyerhof also described the preparation and partial purification of an apyrase from yeast. Steinbach and Moog (12) demonstrated the localization of adenylypyrophosphatase in cytoplasmic granules of chick embryo. By differential high speed centrifugation these authors were able to accomplish a fractionation into ATP-ase and apyrase, the former splitting only one and the latter two phosphate groups. Evidence was not available, however, to decide whether the two were distinct enzyme fractions. By using different extraction media Barth and Jaeger (13) were able to prepare 3 distinct protein complexes from frog's egg, all with apyrase activity, but differing characteristically in relation to thermostability and pH-activity dependence.

Meister (14) reported "adenosinetriphosphatase" activity of human serum; at alkaline pH it was found that all 3 phosphate groups were split from ATP; under similar conditions muscle adenylic acid was completely split. ATP-ase and apyrase activities have been demonstrated in a variety of other metabolizing tissues also, without any serious attempt at purifying either system. A survey of modern literature reveals the fact that there exists considerable confusion in terminology, and that the two terms "apyrase" and "ATP-ase" are often used interchangeably.

It is doubtful whether unequivocal evidence is yet available for characterizing the apyrases as an individual class of enzymes. Apyrases from different sources have been shown to differ with respect to pH optima, heat stability, calcium activation and many other properties.

Steinbach and Moog (12) consider the possibility of apyrase being an enzyme system made up of a combination of ATP-ase and ADP-ase, or of ATP-ase and myokinase, or of ATP-ase and monoesterase and other enzymes. Meyerhof (11) termed his enzyme preparation from yeast an apyrase but reported no data which would show that the purified product split off 2 of the 3 phosphate groups from ATP. Barth and Jaeger (13) also did not furnish any conclusive data to prove that their apyrase preparation split off the two labile groups from ATP. Even Kalekar's evidence for the individuality of potato apyrase does not appear to be very satisfactory. A single experiment seems to have been reported wherein 40 γ of the enzyme were incubated with ATP containing 120 γ of labile phosphorus; at the end of 15 min. 114.5 γ of phosphorus were split, that is, an amount approximating to 2 of the 3 phosphate groups of ATP. To form a complete picture of the extent of splitting of phosphorus from ATP, it would have been desirable to allow the reaction to proceed for a longer period of time to find out whether an endpoint was reached; also, different concentrations of enzyme should have been tried with a given concentration of substrate or *vice versa*. Again, it is to be greatly desired that the final preparations of enzymes should have been tested for the presence of the ordinary phosphatases. Albaum and Kletzkina (15) used apyrase prepared according to the method of Kalekar for establishing the structure of ATP which they isolated from *Drosophila*; their graph shows that, at the end of as long a period as 4 hr. incubation 2 phosphate groups are split from ATP, but no data were given for the concentrations of enzyme or substrate employed. Using the homogenate assay technique of DuBois and Potter, Roberts and Carruthers (16) demonstrated the presence of apyrase in normal epidermis and squamous cell carcinoma; these homogenates brought about the complete dephosphorylation of ATP in 15–45 min.; the activity was not attributable to alkaline phosphatases, since glycerophosphate was not hydrolyzed under the conditions employed. Axelrod (17) reported the purification of a phosphatase from citrus fruit juice with phosphomonoesterase activity; when tested with ATP, the preparation showed "apyrase" activity. The rate of splitting of phosphorus from ATP was very slow, but it would appear from his graph that given sufficient time, complete dephosphorylation of ATP would take place.

From the work mentioned above it was considered that the properties of potato apyrase should be investigated in further detail, especially

with regard to the extent of splitting of phosphate groups from ATP. Three preparations of apyrase were investigated; 1, the initial crude extract obtained by grinding potato in a Waring Blendor with *M*/100 cyanide solution; 2, purified preparations of soluble apyrase obtained by ammonium sulfate fractionation; and, 3, the insoluble apyrase fraction which separates out during dialysis of the precipitates obtained on treatment of potato extract with ammonium sulfate. The preparation of these products has been described elsewhere (18). The final preparations of soluble apyrase, which represent a 1000 to 2700-fold purification on a dry weight basis, seem to be still slightly contaminated with ordinary phosphatases, but the fact that, over a very wide range of dilution of enzyme, in the case of both the soluble and insoluble fractions, 2, and only 2, of the 3 phosphate groups are split when sufficient time is allowed for completion of the reaction, would certainly indicate that the ability to split off 2 phosphate groups from ATP is a characteristic property of apyrase of potato. The observation that, at higher concentrations of the enzyme, all 3 phosphate groups are split off from ATP should probably be attributed to contamination with adenylases.

EXPERIMENTAL

1. *Extent of Splitting of ATP*

A. Crude Potato Extract. An aqueous cyanide extract of potato was prepared and the following system set up, using the undiluted extract and the following dilutions of the same: 1:5, 1:10, 1:25, 1:50 and 1:100.

- 10 cc. enzyme
- 5 cc. *M*/10 succinate buffer, pH 6.5
- 2 cc. calcium chloride, containing 10 mg.
- 2 cc. ATP solution containing 1080 γ of total phosphorus
- 1 cc. water

Total volume 20 cc.

At intervals of 1, 3, 5, 10, 15, 30, 45, and 60 min., 2 cc. aliquots of the reaction mixture were pipetted into excess trichloroacetic acid and the inorganic phosphorus estimated by the method of Sumner (19). The data are presented graphically in Fig. 1, which shows that the undiluted extract splits off all 3 phosphate groups from ATP, and the 1:5 dilution of the enzyme splits 79% of the total phosphorus. The 1:10, 1:25, and 1:50 dilutions of extract apparently split more or less the same amount, representing 2 of the 3 phosphate groups. The shape of the curve with dilution of 1:100 shows that, in this experiment, insufficient enzyme was available to exert maximum activity in the given period of time.

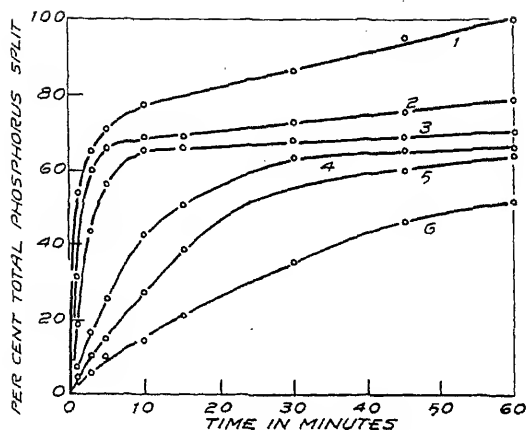


Fig. 1. Splitting of ATP by crude potato extract. 1. Undiluted extract. 2. 1:5 dilution of extract. 3. 1:10 dilution of extract. 4. 1:25 dilution of extract. 5. 1:50 dilution of extract. 6. 1:100 dilution of extract.

B. Soluble Apyrase. The apyrase used in these and the following experiments was obtained by the fractionation procedure outlined under Method 1 in the previous communication (18); the solution was practically colorless, and had a dry weight of 0.3 mg./cc. and an activity of 66,000 units/mg. dry weight.

A system was set up exactly like the one used with crude potato extract. In one experiment the undiluted enzyme solution was used, and in others, 1:25 and 1:50 dilutions of the enzyme solution were employed. The results, graphically represented in Fig. 2, show that, when the undiluted enzyme solution is used, about 66% of the total phosphorus is split at the end of the first min., and that thereafter there is a slow splitting, so that at the end of 60 min. about 94% of the total phosphorus is split. When the enzyme solution is employed in dilutions of 1:25 and 1:50, the reaction proceeds at a rapid rate during the first 2 min., but at the end of 15 min. when $\frac{3}{4}$ of the total phosphorus, that is, all the easily hydrolyzable phosphorus is split, the curve flattens, indicating that further splitting does not occur, even though ATP was present in definite excess.

That this was not due to inactivation of enzyme, and consequent nonavailability for the reaction, was demonstrated by the fact that, when more of the diluted enzyme was added to the reaction mixture at the end of 15 min. time, there was practically no further splitting. The conclusion has, therefore, to be drawn that, in moderate concentrations, soluble apyrase splits off just 2 of the 3 phosphate groups of ATP.

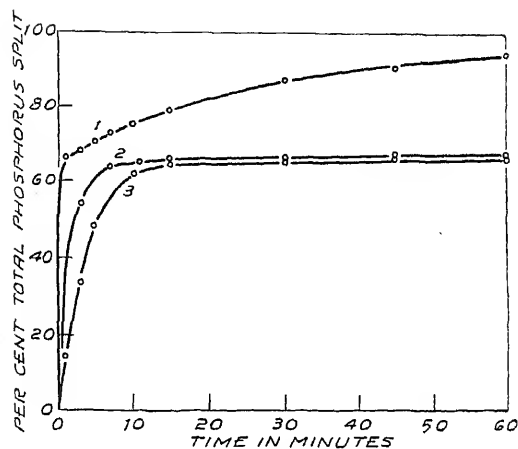


FIG. 2. Splitting of ATP by purified soluble apyrase. 1. Undiluted enzyme solution. 2. 1:25 dilution of the enzyme solution. 3. 1:50 dilution of the enzyme solution.

C. Insoluble Apyrase. It was mentioned previously that, during fractionation of apyrase, a considerable part of the activity passes into the solid fraction that separates during dialysis against distilled water.

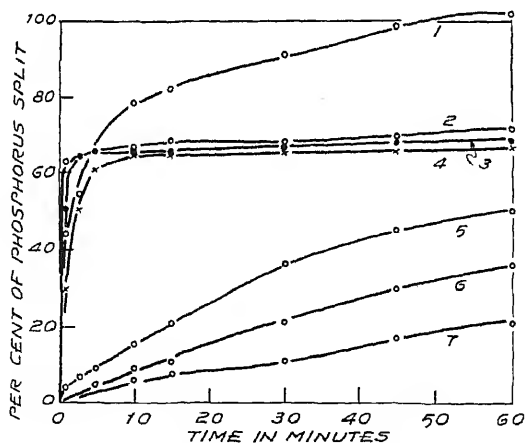


FIG. 3. Splitting of ATP by insoluble apyrase. 1. Concentrated enzyme suspension. 2. 1:5 dilution of enzyme suspension. 3. 1:10 dilution of enzyme suspension. 4. 1:25 dilution of enzyme suspension. 5. 1:400 dilution of enzyme suspension. 6. 1:800 dilution of enzyme suspension. 7. 1:1600 dilution of enzyme suspension.

A sample of this precipitate was washed twice with cold distilled water on the centrifuge and the final residue suspended in water and used in the various experiments. One cc. of the suspension had a dry weight of 22.3 mg. and the activity of the preparation corresponded to 682 units/mg. dry weight.

Fig. 3 illustrates the course of splitting of ATP, using the undiluted suspension and the following dilutions of the insoluble apyrase fraction: 1:5, 1:10, 1:25, 1:50, 1:100, 1:200, 1:400, 1:800, and 1:1600. Since many of the points overlap in the graph, the amounts of phosphorus split at the end of 1 hr. by the various dilutions of the enzyme are separately presented in Table I.

TABLE I
Splitting of Phosphorus from ATP by Insoluble Apyrase

Dilution of enzyme	Total phosphorus split in one hour per cent
Undiluted	102
1:5	72.4
1:10	68.6
1:25	66.7
1:50	66.7
1:100	67.6
1:200	65.7
1:400	49.5
1:800	36.2
1:1600	21.0

The figures in Table I demonstrate clearly that, over a wide range of dilution, namely, 1:10 to 1:200 the enzyme splits almost exactly 2 of the 3 phosphate groups from ATP. The undiluted preparation splits off all 3 phosphate groups, and, in dilutions of 1:400 and above, there is a steady fall in the amount of phosphorus split, and the reaction, although considerably slowed down, still proceeds at a slow rate after the completion of 1 hr.

Fig. 3 also illustrates the principle adopted in estimating the activity of apyrase by suitable dilution. In the tests with the 1:800 and 1:1600 dilutions of enzyme, it is evident that the enzyme is the limiting factor. The amount of phosphorus split at the end of 30 min. by the 1:800 dilution of the enzyme is almost exactly double the amount liberated in the same period by the 1:1600 dilution of the enzyme. Obviously, in the region of these dilutions, there is a strict proportionality between the amount of phosphorus liberated and the concentration of enzyme.

2. pH Optimum

A. *Soluble Apyrase.* A 1:400 dilution of the enzyme solution was used in the standard test and the amount of phosphorus liberated at the end of 30 min. with buffer solutions of different pH was estimated. The results are graphically represented in Fig. 4. It will be seen from the figure that, for the calcium-activated enzyme system, the optimum pH lies in the range 5.5–6.5. When buffers with pH 8 and above were used,

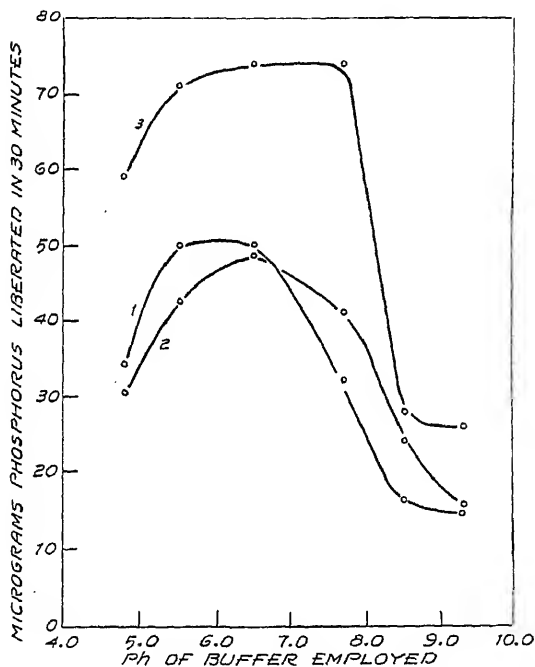


FIG. 4. Relationship between pH and activity of apyrase. 1. Soluble apyrase with added calcium. 2. Soluble apyrase without added calcium. 3. Insoluble apyrase with added calcium.

it was observed that calcium was being precipitated as insoluble phosphate. To make sure that the removal of calcium from the sphere of action did not mask the influence of pH on the speed of reaction, an identical series of experiments was set up where calcium chloride was omitted and water added instead. The graph shows a sharper peak for the system without calcium, but the optimum remains more or less

the same, namely, in the neighborhood of 6.5. (In view of the fact that, when calcium was excluded from the system, the reaction rate was slowed down, a higher concentration of enzyme, namely, 1:100 dilution, was chosen for these tests.)

B. Insoluble Apyrase. A 1:200 dilution of the enzyme was used in the standard test, which contained added calcium. The results shown graphically in Fig. 4 indicate that the enzyme has a fairly wide range of pH, namely 5.5–7.7. At higher alkalinity, the activity decreases rapidly.

3. Activation by Calcium Ions

Calcium is known to be an activator for apyrases. In fact, it has even been suggested that calcium activation may be used in detecting apyrase systems. However, Steinbach and Moog (11) reported that the effect of calcium on the apyrase of chick embryo was highly variable. The effects of the addition of calcium chloride (in the concentrations present in the standard test) on the amount of phosphorus liberated with crude potato extract, soluble apyrase, and insoluble apyrase, are represented in Table II. The dilutions of the enzymes were so chosen that the enzyme was the limiting factor in each test.

TABLE II
Activation of Apyrase by Calcium Ions

	Dilution of preparation	Micrograms of phosphorus liberated in 30 min.	
		With added calcium	Without calcium
A. Crude potato extract	1:50	84	59
	1:100	50	25
	1:200	23	11
B. Soluble apyrase	1:200	88	25
	1:400	54	11
	1:800	27	8
C. Insoluble apyrase	1:200	74	14
	1:400	49	8
	1:800	26	5

The figures in Table II show that calcium has an activating influence on apyrase in all the forms tested. The activation is about 2-fold in the case of the crude extract, about 3- to 4-fold in the case of soluble apyrase and more than 5-fold in the case of insoluble apyrase fraction.

4. Substrate Specificity of Apyrase

The 3 preparations of the enzyme, in dilutions at which they liberate 2 phosphate groups from ATP (as determined by the experiments described in a previous section), were tested against some of the common substrates. A 1:10 dilution of potato extract, a 1:50 dilution of soluble apyrase, and a 1:50 dilution of insoluble apyrase were used in the experiments. The results are recorded in Table III. In the case

TABLE III
Substrate Specificity of Apyrase

Substrate	Total phosphorus in substrate	Phosphorus liberated in 30 min. (γ)		
		Crude extract	Soluble apyrase	Insoluble apyrase
ATP	108, 115, 105	77	75	69
Glucose-1-phosphate	74	traces	nil	nil
Muscle adenylic acid	51	13	2	2
Yeast adenylic acid	66	25	4	2
Sodium pyrophosphate	142	34	8	2
Hexose-1,6-diphosphate	97	23	9	nil
β -Glycerophosphate	76	18	3	nil

of all substrates other than ATP, enough material was used in the test so that the total phosphorus was at least 3 times more than the amount of phosphorus split at the end of the reaction, in order to provide a definite excess.

The figures reported in Table III show clearly that crude potato extract, at a dilution at which 2 phosphate groups are split from ATP, contains phosphatases acting on a variety of substrates. However, in every case, the amount of phosphorus liberated is less than that from ATP, showing thereby that, even in the crude stage, the enzyme shows a degree of specificity toward ATP, or else that the preparation contains mostly apyrase, with smaller amounts of other phosphatases.

The purified soluble fraction shows a high degree of specificity toward ATP, but is still contaminated with small amounts of the ordinary phosphatases. The insoluble fraction of apyrase is remarkably specific toward ATP and appears to be freer from contamination with ordinary phosphatases than the soluble fraction.

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SUMMARY

1. Apyrase activity of crude potato extract, as well as purified soluble apyrase and partially purified insoluble apyrase, is augmented by the presence of calcium ions.

2. Under the conditions of pH obtaining in the standard test a number of ordinary phosphatases present in the crude potato extract can split phosphorus from their specific substrates. The soluble apyrase fraction contains small amounts of ordinary phosphatases, whereas the insoluble fraction seems to be practically free from contamination with these phosphatases.

3. Both the soluble and insoluble fractions of apyrase behave identically with respect to pH optimum, activation by calcium ions, splitting of ATP and substrate specificity.

4. In concentrated form apyrase appears to split all the phosphate groups from ATP, probably due to contamination with adenylases, but over a considerable range of dilution the enzyme splits exactly 2 of the 3 phosphate groups.

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The Role of Pyruvate in the Aerobic Respiration of Barley Roots

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INTRODUCTION

Although a great deal of attention has been given the glycolytic paths and mechanisms involved in the respiration of higher plants, comparatively little information has been vouchsafed regarding the aerobic metabolism of the 3-carbon products of the glycolytic process. Investigations, carried out both with microorganisms and a variety of animal tissues, have indicated pyruvate to be a critical intermediate in the oxidation of carbohydrate. Although pyruvate is known to be present in higher plants, there is little evidence to indicate the extent or manner of its aerobic utilization.

James *et al.* (18) have demonstrated the formation of pyruvate in barley shoots, while Bunting and James (7) have demonstrated the decarboxylation of the latter to acetaldehyde. The fate of the 2-carbon product of pyruvate decarboxylation has not been described. The role of certain dicarboxylic and tricarboxylic acids in higher plant respiration has been dealt with by Machlis (24), Henderson and Stauffer (14), Bonner and Wildman (6) and others. A thorough review has been presented by James (17). It is the purpose of the present investigation to adduce additional information pertaining to the aerobic metabolism of pyruvate in higher plants, and to ascertain to what extent the experimental findings coincide with the tricarboxylic acid cycle theory proposed by Krebs and Johnson (20).

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MATERIALS AND METHODS

The barley used throughout this investigation was of the variety "Sacramento," grown at Davis, Calif. The germinating, planting, and preparative procedure followed that of Machlis (24), with the exception that weak CaSO_4 solution (0.0025 M) was substituted for simulated tap water during the growth period. Although roots grown in glass-distilled water were obviously abnormal, no differences were apparent between roots grown in CaSO_4 solution and roots grown in full nutrient solution. Sixty 15 mm. segments were counted into separate vials containing either phosphate buffer or experimental solutions. After a 2 hr. soaking period the segments were transferred to respirometer cups containing 2 ml. of liquid.

Gas Exchange Measurements

Oxygen uptake was measured by the "Direct Method" of Warburg (29). When additions were made from the sidearm, 1 ml. of solution in the sidearm was added to 2 ml. of solution in the main chamber. Respiratory measurements were carried out for 5-8 hr. after equilibration. Machlis has indicated that microbial contamination in the presence of 1% glucose is negligible for at least 12 hr.

All experimental solutions were 0.01 M with respect to phosphate, and adjusted to pH 5.0 with NaOH or HCl . Whenever the sodium or potassium salt of an organic acid was used in an experiment, those root segments not receiving organic acid were provided with an equivalent amount of salt in the form of Na_2SO_4 or K_2SO_4 . In the calculation of vessel constants the average volume of 60 root segments, 162 mm^3 , was computed in terms of water.

Respiratory quotients were determined by the "Direct Method" of Dixon (11). The reproducibility of rates of oxygen uptake by equal numbers of barley root segments has been shown to be such that little error is to be expected in the calculation of respiratory quotients by the above method (Machlis).

Respiratory Characteristics of Excised Barley Roots in the Presence of Glucose and Pyruvate, Respectively

Studies were carried out both with normal and depleted tissue, with the end in view of obtaining larger respiratory responses to substrate addition where carbohydrate reserve was first diminished. Roots were depleted by immersion, immediately after excision, in 0.0025 M CaSO_4 solution, wherein the tissue was aerated for a 24 hr. period. At the end of this time the roots were segmented in the usual manner and respiration measurements were carried out. Results are presented in Table I.

In both normal and depleted tissue the rate of respiration in the presence of pyruvate was sufficiently high to allow the consideration of this compound as an intermediate product of carbohydrate breakdown. As indicated in Table II, pyruvate concentrations greater than 0.05 M proved deleterious. The diminution of respiration at higher pyruvate concentrations probably represents injury due to high salt concentration, since equivalent amounts of Na_2SO_4 bring about the same results.

TABLE I
*Respiration of Normal and Depleted Barley Root Segments in the
 Presence of Glucose and Pyruvate, Respectively*

Substrate	Normal			Depleted		
	mm. ³ O ₂ /60 seg./hr.		Per cent of control	mm. ³ O ₂ /60 seg./hr.		Per cent of control
	Control	Substrate added		Control	Substrate added	
2% Glucose	35.9	47.2	132	17.9	31.5	176
0.05 M Pyruvate	38.7	47.6	123	20.1	28.8	141

Although the percentage increases in respiration were in all cases much larger in depleted roots than in normal roots, the absolute rates of oxygen uptake remained higher in normal roots. Since, in addition, depleted roots appeared more sensitive to injury from high salt concentrations than normal roots, unstarved roots were used in all subsequent experiments in an effort to maintain the tissues as near to their normal state as possible.

TABLE II
Effect of Pyruvate Concentration on Respiration of Depleted Root Segments

Concentration	mm. ³ O ₂ /60 seg./hr.	Per cent of Control
Control (0.025 M Na ₂ SO ₄)	24	100
0.005 M Pyruvate	28	114
0.025 M Pyruvate	31	129
0.05 M Pyruvate	37.5	156
0.1 M Pyruvate	17.4	73
0.05 M Na ₂ SO ₄	17.2	72

Penetration of substrate into the tissue is rapid. Segments placed in pyruvate solutions immediately prior to respiratory measurements respired as actively at the end of the first 15 min. period of measurement as those segments receiving substrate during the entire 2 hr. preexperimental period.

*The Effect of Certain Enzyme Inhibitors and Metabolic
 Intermediates on the Respiration of Barley Roots*

Iodoacetic Acid. The extent to which barley root respiration is inhibited by iodoacetic acid is related to the pH. This relationship was

observed by varying the pH in a system comprised of root segments in a solution of phosphate buffer and $4 \times 10^{-5} M$ moniodoacetic acid (Fig. 1). The change in magnitude of inhibition with changing pH is quite marked, there being approximately 75% inhibition at pH 5.0,

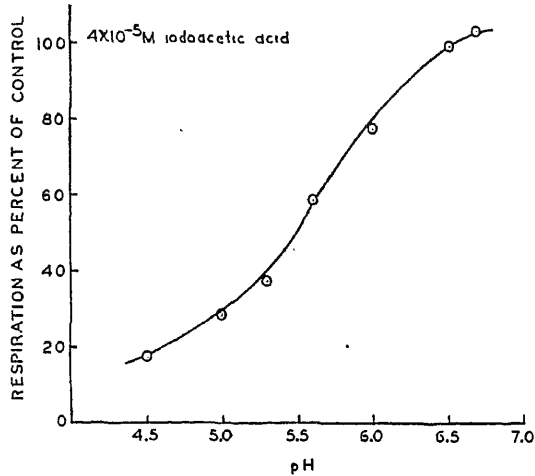


FIG. 1. The inhibition of barley root respiration by iodoacetic acid as a function of pH.

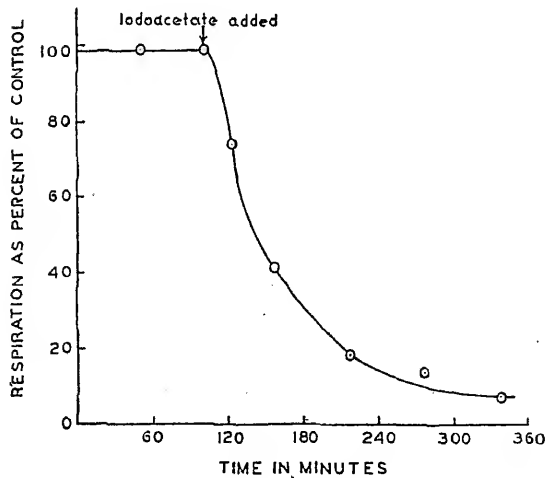


FIG. 2. The inhibition of barley root respiration by iodoacetic acid as a function of time. Iodoacetic acid concentration: $4 \times 10^{-5} M$

and no inhibition at pH 6.5. It appears likely that the pH effects the penetration of the inhibitor rather than its action within the cell. The rapidity with which iodoacetic acid inhibition takes place and the course such an inhibition follows with time is graphically depicted in Fig. 2.

Iodoacetic acid has been shown to inhibit several dehydrogenases (1,2,9,10). However, at sufficiently low concentrations inhibition has been ascribed mainly to the inactivation of 1,3-diphosphoglycerate dehydrogenase (1). Addition of pyruvate to such an inhibited system should permit the respiratory process to continue.

Root segments previously inhibited with iodoacetate were transferred to a solution of iodoacetate and pyruvate. Alternatively, uninhibited root segments were placed immediately in a solution of iodoacetate and pyruvate. The respiration of segments in the presence of pyruvate was compared to that of segments placed in iodoacetate alone (Table III). Comparable experiments were carried out in which substrate was added from the sidearm to inhibited root segments (Fig. 3).

TABLE III
Effect of Pyruvate on Iodoacetate-Inhibited System

	Control	mm. ³ O ₂ /60 segments/hr.		Iodoacetate + 0.05 M pyruvate	Per cent of control
		4 × 10 ⁻⁵ M Iodoacetate	Per cent of control		
Substrate added after inhibitor	44.0	23.0	52	28.0	64
Substrate added with inhibitor	41.1	18.4	45	40.5	98

It is evident from the preceding data, that pyruvate exerts a much greater respiratory effect when added concomitantly with iodoacetic acid than when added in a subsequent period. The work of Barron and Singer (5), and Hopkins *et al.* (15), indicates a possible interpretation of the observed phenomenon. These authors have shown that both succinate and malonate protect succinic dehydrogenase, a sulfhydryl enzyme, from the action of -SH inhibitors. Other enzyme systems (pyruvate oxidase, malic dehydrogenase) have also been characterized as sulfhydryl enzymes susceptible to inhibition by iodoacetate. The addition of pyruvate together with the inhibitor may protect one or more enzymes from inactivation, either indirectly by the formation of succinate, or perhaps directly by the protection of pyruvate oxidase.

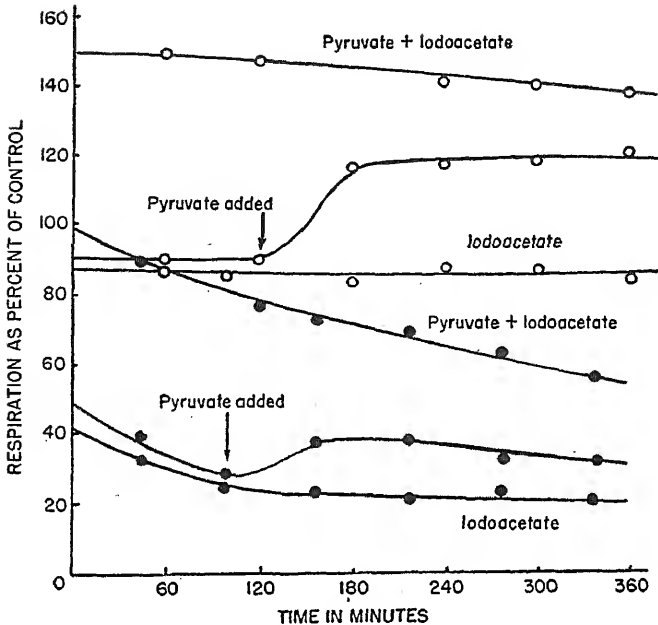


FIG. 3. The effect of pyruvate on the respiration of barley roots inhibited with iodoacetic acid. Open circles: 1×10^{-5} *M* iodoacetic acid. Solid circles: 4×10^{-5} *M* iodoacetic acid. Curves marked "Pyruvate + Iodoacetate" represent respiration of segments which received substrate and inhibitor concomitantly. "Pyruvate added" indicates pyruvate addition to inhibited root segments. Pyruvate concentration: 0.05 *M*.

Massart and van den Noortgaete (25), and Thimann and Bonner (28) have observed comparable effects in yeast respiration and *Avena* growth respectively. Once inhibition by iodoacetate has been achieved, subsequent additions of substrate are without effect (5).

It is interesting to consider, at this point, the possibility that malonate may, when used in conjunction with iodoacetic acid, appear to stimulate respiration, when in actuality this substance may simply protect the enzyme against the more drastic inactivation effected by iodoacetate.

When pyruvate is added previous to the inhibitor, its effect is still manifest upon the addition of inhibitor. In the following experiment pyruvate was permitted to infiltrate the roots in the first preliminary

period. In the second preliminary period the segments were placed in iodoacetic acid. In the experimental period root segments received both pyruvate and iodoacetate. With this treatment respiration was inhibited 16% in the experimental period instead of 40–60% as observed in previous experiments. When pyruvate alone was added in the preliminary period, a stimulation was observed (Table IV). A fundamental role of pyruvate in the respiration of barley roots is indicated whether the action of pyruvate in maintaining respiration in the presence of iodoacetate is attributed to the reestablishment of a metabolic chain interrupted by the inhibition of phosphoglyceraldehyde dehydrogenase, or whether pyruvate is considered a protective substrate, or protective substrate precursor.

TABLE IV
Effect of Pretreatment of Iodoacetate-Inhibited System on the Subsequent Response to Pyruvate Addition

Treatment in preliminary periods		mm. ³ O ₂ /60 segments/hr.		
Period I	Period II	Control	4 × 10 ⁻⁵ M Iodoacetate + 0.05 M pyruvate	Per cent of control
0.05 M Pyruvate	4 × 10 ⁻⁵ M Iodoacetate	36.5	30.6	84
0.025 M Na ₂ SO ₄	0.05 M Pyruvate	36.5	38.8	106

Fluoride. Fluoride is known to inhibit enolase (23,30), as well as animal, yeast, and higher plant phosphatase (3,26,31). If the action of fluoride in the following experiments were primarily upon the phosphatases, the addition of pyruvate would not result in increased respiratory activity. Since pyruvate does reestablish respiration, the action of fluoride on the enzyme enolase is indicated.

In the following experiment, root segments were divided into 2 groups, one of which received fluoride in a preliminary period while the other received both fluoride and pyruvate. The fluoride-treated segments were, in turn, divided during the experimental period, one group being placed once again in fluoride solution, the other group being placed in a solution of fluoride and pyruvate. Fig. 4 depicts the magnitude of fluoride inhibition as well as the ability of pyruvate to reverse this inhibition. Pyruvate, whether added during the preliminary period, or at the beginning of the experimental period, is metabolized, and respiratory rates approximating the control are achieved.

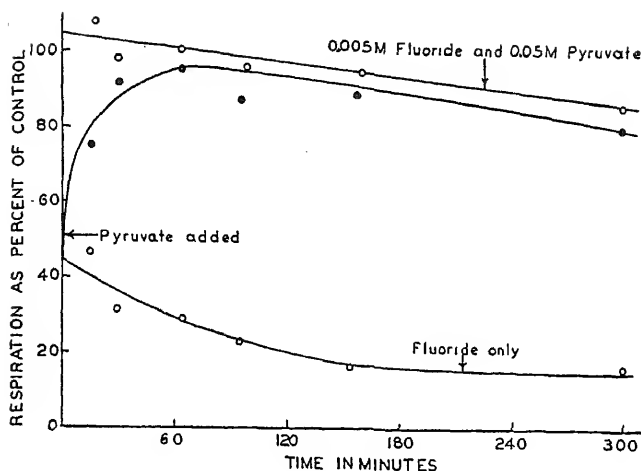


FIG. 4. The effect of pyruvate on the respiration of barley roots inhibited with NaF. Curve marked "Fluoride and Pyruvate" represents respiration of segments which received substrate and inhibitor concomitantly. Curve marked "Pyruvate added" indicates pyruvate addition to inhibited root segments.

An attempt was made to derive the respiratory quotient specifically associated with the metabolism of pyruvic acid by subtracting the quantities of gas exchanged in the presence of buffer, or buffer and fluoride, from the quantities of gas exchanged in the presence of pyruvate under respectively similar conditions. The respiratory quotient calculated from the observed differences was assumed to be that for pyruvate metabolism. When the respiratory quotient directly associated with pyruvate metabolism is calculated as described above, the anomaly is encountered whereby the R. Q. for pyruvate oxidation in the presence of fluoride is 1.09 or lower, whereas the R. Q. for the oxidation of the same substrate in the absence of fluoride is 1.37 or higher (Table V).

The overall R. Q. is higher in the presence of fluoride than in the presence of fluoride and pyruvate together. The high R. Q. in the presence of fluoride, compared to that for the control, suggests organic acid oxidation. When the source of pyruvate is unimpaired, the oxidation of added organic acid is not accompanied by a rise in R. Q. (see "Malonic Acid"). It would appear that, in the absence of pyruvate, the 4-carbon acids are oxidized directly, whereas in the presence of pyruvate, the same acids take part in the oxidation of pyruvate in a manner described by Krebs and Eggleston (21). Therefore, in the pres-

TABLE V
*Respiratory Quotients Related to Pyruvate Metabolism in the
 Presence and Absence of Fluoride (0.005 M)*

Expt.	Treatment	mm. ³ Gas/60 segments		Overall R. Q.	mm. ³ Gas in excess of control		R. Q. from gas exchange in excess of control
		O ₂	CO ₂		O ₂	CO ₂	
1	Control	41.5	32	0.77	—	—	—
	0.05 M Glucose	49.6	40.6	0.82	8.1	8.6	1.06
	0.05 M Pyruvate	45.6	43.6	0.96	4.1	11.6	2.84
2	Depleted tissue control	20.2	10.5	0.52	—	—	—
	0.05 M Glucose	29.4	18.5	0.63	9.2	8.0	.87
	0.05 M Pyruvate	37.4	34.9	0.93	17.2	24.4	1.42
3	0.005 M NaF	45.0	53.0	1.18	—	—	—
	NaF+0.005 M Pyruvate	77.0	85.0	1.10	32	32	1.00
	NaF+0.025 M Pyruvate	89.0	101.0	1.12	44	47.9	1.09
4	0.005 M NaF	28.6	30.4	1.06	—	—	—
	NaF+0.005 M Pyruvate	56.6	50.6	0.89	28.0	20.2	0.72
	NaF+0.05 M Pyruvate	72.0	70.0	0.97	43.4	39.6	0.91
5	Control	66.0	56.0	0.85	—	—	—
	0.05 M Pyruvate	104	108	1.04	38	52	1.37
	NaF+0.05 M Pyruvate	62.5	68.2	1.10	—	—	—

ence of fluoride, the calculation of the R. Q. associated with pyruvate metabolism by the above method will yield a low value.

The excessively high R. Q. for pyruvate metabolism in the absence of fluoride may be the result of vigorous carboxylase activity (4,16), oxidative assimilation (8), or the selective oxidation of pyruvate as described by Krebs and Eggleston. If selective oxidation of pyruvate occurs, and an R. Q. for pyruvate oxidation is formulated from the difference of the endogenous gas exchange and the gas exchange observed in the presence of pyruvate, the R. Q. thus calculated will be higher than the theoretical for complete oxidation of pyruvate.

Malonic Acid. The effect of pH on the inhibition of barley root respiration by malonate is closely analogous to the effects observed in the presence of iodoacetic acid. Inhibition is appreciable at pH 4.0, and not at all evident at pH 6.0 (Fig. 5). That the pH effect is associated

with the presence of the inhibitor is clearly evident from the lack of response of endogenous respiration to a change in pH. Similar relationships were observed in spinach (6).

Although inhibition was found to increase with increasing malonate concentration up to 0.05 *M*, 0.01 *M* malonate was used to avoid excessively high salt concentrations when substrates were added in addition to the inhibitor. The extent of malonate inhibition as a function of time is depicted in Fig. 6. Noticeable inhibition takes place in one hr., and maximum inhibition takes place in approximately 1.5 hr.

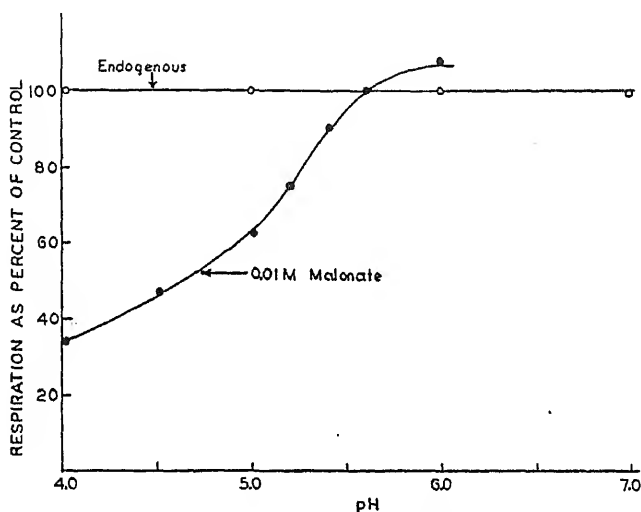


FIG. 5. Inhibition of barley root respiration by 0.01 *M* malonate as a function of pH. Endogenous respiration determined in the presence of 0.01 *M* phosphate buffer.

Subsequent experiments will show that at pH 5.0, inhibition, once achieved, does not remain constant, but rather falls off gradually with time. In 12 experiments, the average decrease of inhibition over a 5 hr. experimental period was approximately 28%. At pH 4.5, however, inhibition remains constant. The diminution of inhibition with time at pH 5.0 may be due to the increase of succinate concentration within the cell. Such an increase will be demonstrated in a later paper. At pH 4.5, although succinate may be accumulating, the larger malonate concentration within the cell, brought about by its more ready penetration at the lower pH, still prevents the oxidation of succinic acid.

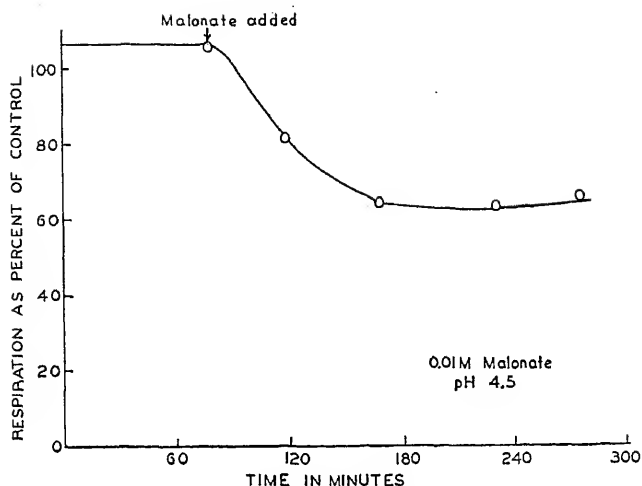


Fig. 6. Inhibition of barley root respiration by 0.01 *M* malonate as a function of time.

*The Effect of Pyruvate and Certain Dicarboxylic Acids on
Malonate-Inhibited Barley Roots*

In the following series of experiments, glucose, pyruvate, acetate, citrate, *cis*-aconitate, α -ketoglutarate, succinate, fumarate, and *l*-malate respectively were added to malonate-inhibited systems. The effect of these substrates on the respiration of such inhibited systems is summarized in Table VI. In the experimental treatments involving citrate and *cis*-aconitate, root segments were transferred directly from malonate solutions into the experimental solutions, no additions being made from the sidearm. With the exception of acetate, all the added substrates reestablished respiration at levels equivalent to, or exceeding that of the control. Acetate caused immediate and continued deleterious effects.

Inhibition by acetate does not appear to be a function of hydrogen ion, since the data in Table VII indicate some inhibition at pH 7.0. If, perhaps, one of the first products of pyruvate oxidation is acetyl-phosphate or a related compound (22), acetate may conceivably inhibit the subsequent utilization of this compound in the same sense that glucose inhibits the sucrose phosphorylase described by Doudoroff (12), namely, by replacing the active form of the substrate at the enzyme surface.

The respiration of root segments in 0.05 *M* citrate or *cis*-aconitate soon fell below that of the malonate-inhibited system. Diminution of respiration in this case appeared to be a salt effect since the respiratory rate of segments in 0.15 *M* KBr, in the absence of inhibitor, was but 50–60% of the control.

TABLE VI
The Effect of Malonate and Certain Organic Acids on the Respiration of Excised Barley Roots

Expt.	Treatment	Control rate mm. ³ O ₂ /60 segs./hr.	Respiration as per cent of control at time of substrate addition	Respiration as per cent of control during successive periods after substrate addition				
				1 hr.	2 hr.	3 hr.	4 hr.	5 hr.
1	0.01 <i>M</i> Malonate	41	60	65	73	82	—	—
	Malonate+0.05 <i>M</i> Glucose	41	68	81	98	115	—	—
2	0.01 <i>M</i> Malonate	35	55	55	65	82	—	—
	Malonate+0.05 <i>M</i> Pyruvate	35	47	64	82	102	—	—
	Malonate+0.01 <i>M</i> Fumarate	35	53	70	94	123	—	—
	Malonate+0.05 <i>M</i> Pyruvate +0.05 <i>M</i> Fumarate	35	55	81	106	129	—	—
3	0.01 <i>M</i> Malonate	34	58	60	65	73	80	82
	Malonate+0.05 <i>M</i> Succinate	34	63	77	104	119	131	140
	Malonate+0.05 <i>M</i> α -Ketoglu- tarate	34	61	73	91	100	108	111
	Malonate+0.05 <i>M</i> Acetate	34	53	33	13	6	4	3
4	0.01 <i>M</i> Malonate	36	55	57	58	60	61	—
	Malonate+0.05 <i>M</i> <i>L</i> -Malate	36	58	88	95	100	105	—
5	0.01 <i>M</i> Malonate	41	48	48	50	56	63	70
	Malonate+0.02 <i>M</i> Citrate	41	—	110	122	127	129	130
	Malonate+0.02 <i>M</i> <i>cis</i> -Aconi- tate	41	—	95	107	115	121	128

Malonate affects both the uptake of oxygen and the release of CO₂ (Table VIII). The fact that the R. Q. in the presence of malonate and fumarate is very close to the R. Q. for the control, and the oxygen uptake in the presence of malonate and fumarate is even greater than

that for the control, is a favorable indication that fumarate enters into a cycle involved in the metabolism of cellular substrate, and is not, in itself, metabolized through a different path.

TABLE VII
*The Effect of Acetate at Different pH Values;
With and Without Malonate*

Treatment	pH	Rate (3d hr.) mm. ³ O ₂ /60 seg./hr.	Percent of control
Control	5.0	43.3	100
0.01 M Malonate	5.0	17.9	41
0.03 M Acetate	5.0	0	0
	6.0	17.0	39
	7.0	34.4	80
0.03 M Acetate + 0.01 M Malonate	5.0	3.2	7
	6.0	9.6	22
	7.0	36.8	85

TABLE VIII
*Effect of Malonate and Fumarate on the Respiratory
Quotient of Barley-Root Respiration*

Treatment	Gas exchange in mm. ³ O ₂ /60 seg./hr.				
	Oxygen uptake	Per cent of control	CO ₂ evolution	Per cent of control	R. Q.
Control	39	100	32	100	0.82
0.01 M Malonate	29.5	76	30.4	95	1.03
0.01 M Malonate +					
0.03 M Fumarate	40	103	31.4	98	0.79

DISCUSSION

The response of malonate-inhibited tissue to the addition of certain dicarboxylic acids should be an indication of whether these acids function merely as hydrogen mediators, as suggested by Szent-Györgyi (27), or whether they function as intermediates in the more

inclusive scheme developed by Krebs. Although an inhibitory effect of malonate is to be expected if a cycle exists, as well as if succinate oxidation occurs as a step in hydrogen transport from substrate to terminal oxidase, the reestablishment of respiration by the addition of fumarate, malate, citrate, *cis*-aconitate or α -ketoglutarate would be expected only if a cycle exists, since the reductive formation of succinate is precluded in the presence of malonate (32). Any consideration of a tricarboxylic acid cycle includes the participation of pyruvate, or a pyruvate derivative. It has been demonstrated that pyruvate is readily utilized by barley roots.

The addition of postulated intermediates of the tricarboxylic acid cycle to newly excised roots brings about but limited increases in respiration, whereas, in the presence of malonate, these same substances elicit a large respiratory response. If added organic acids were being metabolized through pathways other than those of the endogenous respiration, it would be expected that, in uninhibited tissue, the oxygen uptake would be additive. This is not the case, and it appears that malonate limits the natural supply of these organic acids, and this limitation is overcome by addition of the latter to the medium.

Whether or not the 4-carbon acids act catalytically when added to an intact system does not appear to be a suitable criterion for their participation in either a tricarboxylic acid cycle, or a scheme such as presented by Szent-Györgyi. A catalytic effect would be expected only if the concentration of the organic acid were limiting respiration.

Henderson and Stauffer found malonate to be without effect in tomato roots. Failure to achieve inhibition may have been due to the pH employed (5.2–5.8) since data presented in this paper, and corroborated by Bonner and Wildman, indicate little malonate inhibition at pH 5.8. In studies carried out by the same investigators on the effects of certain Krebs cycle intermediates on the respiration of both uninhibited, and fluoride or iodoacetate-inhibited tomato roots, substrate concentrations were frequently 0.1 *M* or 0.00001 *M*. These concentrations of organic acid seem too high and too low, respectively, and may explain the absence of any observable stimulation. Machlis was able to maintain respiration in barley roots in the presence of iodoacetate by additions of citrate or the 4-carbon dicarboxylic acids. No explanation is apparent for his failure to reverse malonate inhibition with the same substrates. Bonner and Wildman, however, reversed fluoride inhibition

in spinach leaves by the addition of pyruvate, and malonate inhibition by the addition of succinate, fumarate, citrate, malate, or pyruvate.

If the theoretical R. Q. for the complete oxidation of a 4-carbon dicarboxylic acid is observed, no indication is given that the acid is not being metabolized through a cycle. The reversible decarboxylation of oxaloacetate has been established (13,19). A molecule of pyruvate resulting from such decarboxylation may combine with another molecule of oxaloacetate to perpetuate the cycle. Neither in the work herein presented, nor in that of Machlis, or of Wildman and Bonner, is the metabolism of Krebs cycle intermediates commonly accompanied by an R. Q. higher than 1.0. Bennet-Clark and Bexon venture an explanation for the high R. Q. observed upon the addition of malate or succinate to beet discs, and do not consider their observations incompatible with the existence of a tricarboxylic acid cycle.

SUMMARY

The rate of oxygen uptake in the presence of pyruvate was comparable to the rate observed in the presence of glucose in both normal and depleted roots. The respiration of excised barley roots was inhibited by moniodoacetic acid and fluoride, respectively. The addition of pyruvate to such inhibited systems reestablished respiration at a level comparable to that of the uninhibited control. When inhibition was achieved by the use of moniodoacetic acid, pyruvate enhanced respiration only when added previous to, or concomitantly with, the inhibitor. Inhibition by fluoride resulted not only in a diminution of respiration, but also in a qualitative change of the residual respiration.

Inhibition of respiration was also effected by malonate. The addition of glucose or pyruvate, as well as citrate, *cis*-aconitate, α -ketoglutarate, succinate, fumarate, and *L*-malate, respectively, reestablished respiration at a level equivalent to, or greater than, that of the uninhibited control.

Acetate produced an immediate deleterious effect on barley root respiration, which effect was manifest throughout a range of acetate concentrations and pH values.

Respiratory quotients accompanying the metabolism of fumarate in the presence of malonate were approximately the same as the respiratory quotients for endogenous respiration.

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Rous Chicken Sarcoma as a Source for Hyaluronic Acid

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INTRODUCTION

The increasing importance attached to hyaluronidase has served to bring into greater prominence the role of hyaluronic acid as the substrate for measuring the activity of the enzyme. The principal sources for the production of hyaluronic acid have been limited to umbilical cord and vitreous humor. Kabat (1) and Pirie (2) showed that a mucinous polysaccharide isolated from fowl sarcoma was similar to, if not identical with, hyaluronic acid. The need for large amounts of this polysaccharide led to investigation of the Rous chicken sarcoma as a practicable source for the production of hyaluronic acid. This paper shows that substantial amounts of hyaluronic acid can be obtained from chicken tumor, and also presents some comparative studies with hyaluronic acid isolated from umbilical cord.

MATERIALS

Plymouth Rock pullets (1.1–1.4 kg.) were injected in each side of the breast with 1 ml. amounts of a standardized suspension of macerated Rous tumor.¹ Twelve to 17 days after inoculation the fowls were sacrificed and a highly mucilaginous tumor tissue removed and stored in acetone. The amount of tissue per chicken ranged between 40 and 100 g., with an average of 60 g.

The polysaccharide was purified by the method of Hadidian and Pirie (3). The yield of the hyaluronic acid from 500 g. of tumor tissue was about 0.5 g. Nitrogen, determined by the micro Kjeldahl method was 3.8%. The tumor hyaluronic acid produces approximately $\frac{1}{3}$ the absolute viscosity of an equivalent amount of umbilical cord hyaluronic acid.

Testicular hyaluronidase, prepared by the method of Madinaveitia (4), was used in the hydrolysis studies. The enzyme preparation contained approximately 125 turbidity reducing units/mg. as evaluated by the assay method of Kass and Seastone (5).

¹ The tumor virus was kindly supplied by Dr. F. Duran-Reynals of Yale University.

Relationship of Turbidity to Hyaluronic Acid Concentration

Meyer and Palmer (6) made the observation that the turbidity produced by the interaction of hyaluronic acid and acidified blood serum is proportional to hyaluronic acid concentration. The comparative turbidimetric properties of tumor and umbilical cord hyaluronic acid were determined.

Various concentrations of each polysaccharide were distributed in a series of tubes, 100×13 mm., and the volume adjusted to 1 ml. with 0.1 *M* acetate buffer pH 6.0. To each tube was added 3 cc. of 0.5 *M* acetate buffer pH 4.2 and 1 cc. of 1:10 dilution of horse serum pH 4.2. The tubes were thoroughly mixed and incubated in a 24°C. water bath for 30 min. Turbidities were read with the aid of a Klett-Summerson photoelectric colorimeter using a red filter No. 66 with an approximate spectral range of 640–700 $m\mu$.

The relationship of turbidity development to concentration of purified tumor and umbilical cord hyaluronic acids are shown graphically in Fig. 1. It is evident that the polysaccharides are very similar, except that the tumor polysaccharide is somewhat less pure.

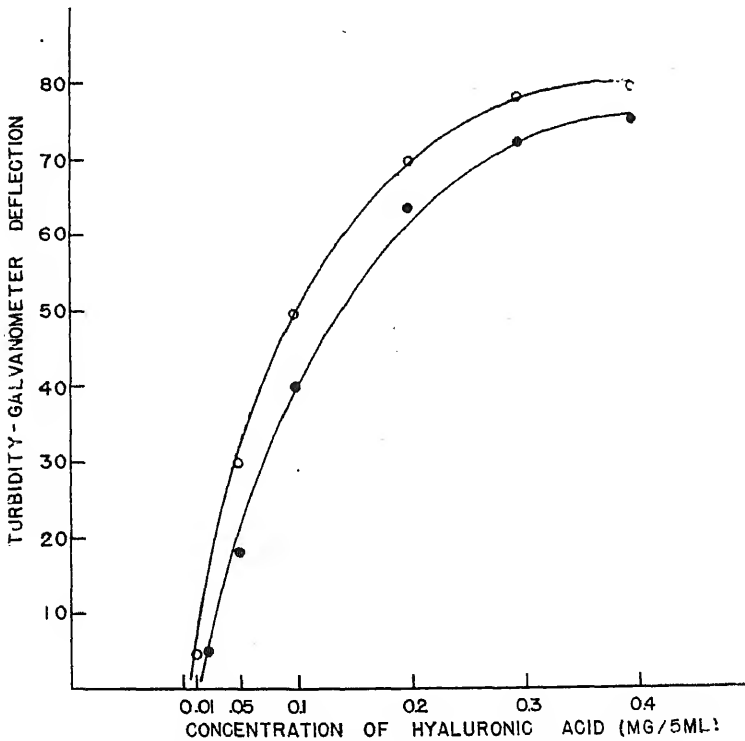


FIG. 1. Relationship of hyaluronic acid to turbidity value.

○—○ = umbilical cord. ●—● = tumor.

The Hydrolysis of Hyaluronic Acid by Testicular Hyaluronidase

The effect of hyaluronidase on the hydrolysis of hyaluronic acid was measured turbidimetrically by the method of Leonard *et al.* (7). The hydrolysis of purified hyaluronic acids isolated from tumor and umbilical cord were compared, the same enzyme dilutions and concen-

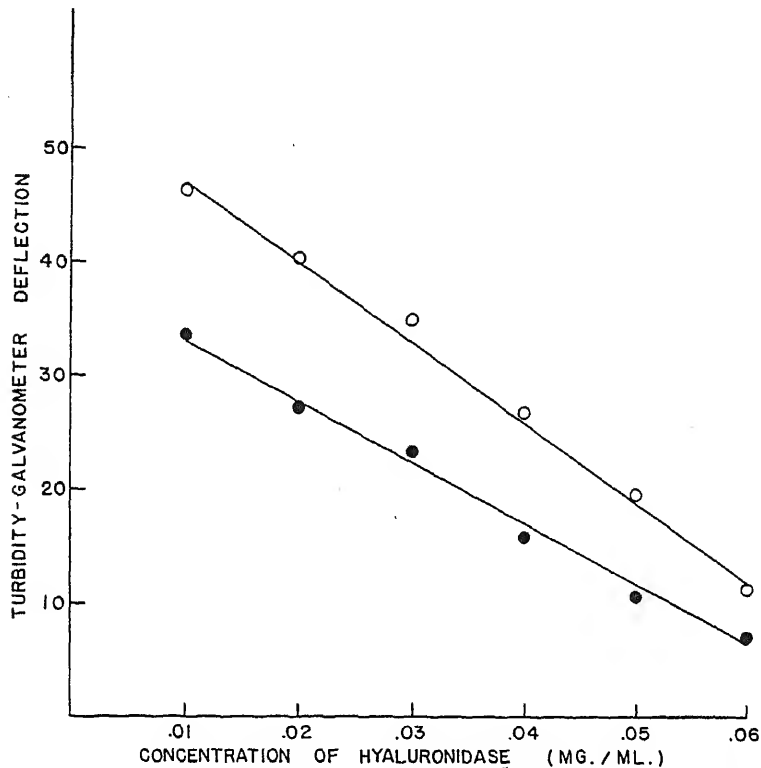


FIG. 2. The hydrolysis of hyaluronic acid by hyaluronidase. ○—○ = Umbilical cord. ●—● = tumor. Each system consisted of 0.5 ml. of 0.4 mg./ml. Hyaluronic acid and 0.5 ml. of various dilutions of hyaluronidase. Incubated 30 min. at 37°C. Reaction stopped and turbidity developed by addition of 3 ml. of 0.5 *M* acetate buffer, pH 4.2, and 1 ml. of 1:10 acidified horse serum, pH 4.2. Read in a Klett-Summerson colorimeter; red filter no. 66.

tration of substrate being employed in this study (Fig. 2). A linear correlation is found between the enzymatic activity of the enzyme dilutions as measured by the hydrolysis of the two substrates. The

higher turbidity values of umbilical cord hyaluronic acid reveal, as in Fig. 1, a higher degree of purification in comparison with tumor hyaluronic acid.

It was thought of interest to follow the degradation of the tumor and umbilical cord hyaluronic acids by using a viscosimetric method. The viscosity reduction of the 2 substrates by hyaluronidase was measured by the method of Haas (8). For the purpose of comparison, optimum concentrations of enzyme and substrate were studied. The curves obtained under these conditions indicate that a higher initial concentration of tumor hyaluronic acid is required to give a viscosity value comparable to that of umbilical cord hyaluronic acid. However, the

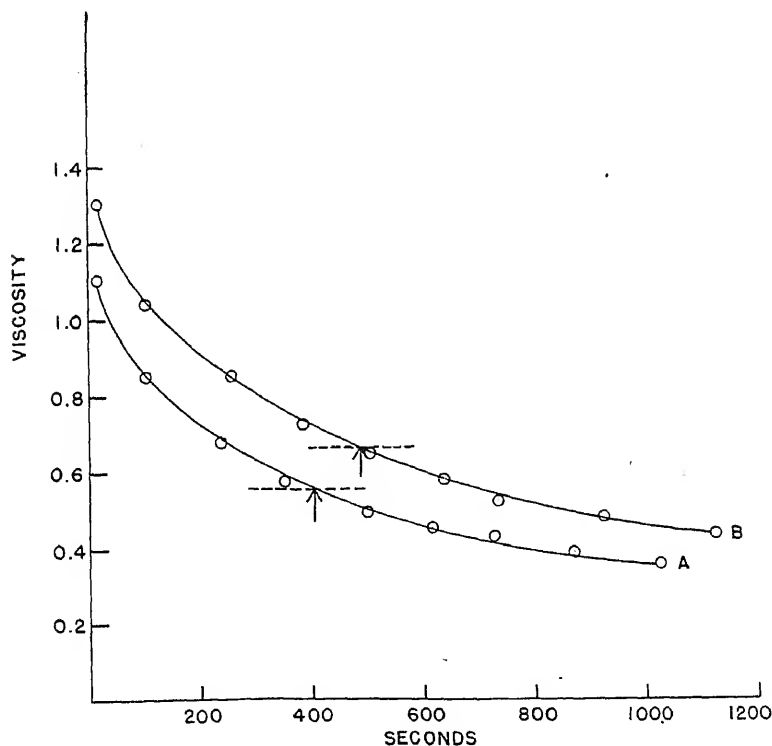


FIG. 3. Sample viscosity reduction assay curves. A = Tumor hyaluronic acid, 1.15 mg./ml.; hyaluronidase, 0.043 mg./ml. B = Umbilical cord hyaluronic acid, 0.50 mg./ml.; hyaluronidase, 0.015 mg./ml. Broken lines indicate half viscosity level. Viscosity expressed as (viscosity of solution/viscosity of buffer)—1.

latter substrate only needed $\frac{1}{3}$ the concentration of hyaluronidase to produce a viscosity reduction value on the same order as that of tumor polysaccharide (Fig. 3).

SUMMARY AND CONCLUSIONS

1. Hyaluronic acid isolated from Rous chicken sarcoma has been shown to serve as a useful substrate for the assay of hyaluronidase.

2. When subjected to the same purification procedure, tumor and umbilical cord gave similar yields of hyaluronic acid.

3. The hydrolysis of tumor and umbilical cord substrates by hyaluronidase reveals that a more highly purified product is obtained from umbilical cord. Nevertheless, the reaction velocities vary linearly with hyaluronidase concentrations in the presence of both substrates.

4. The viscosimetric measurement of the degradation of the two substrates by hyaluronidase shows that approximately a two-fold concentration of tumor hyaluronic acid is required to attain the initial viscosity value of umbilical cord hyaluronic acid. However, the latter substrate needed one-third the concentration of enzyme to produce a viscosity reduction curve on the same order as that of tumor hyaluronic acid.

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Excretion of Amino Acids by Mice Fed Certain Deficient Diets ¹

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INTRODUCTION

In previous studies it was observed that mice fed diets deficient in tryptophan or methionine excreted abnormally high amounts of the ingested amino acids in the urine as compared to mice fed diets containing casein (1). Conversely, the ingestion of proteins of higher biological value than that of casein resulted in the excretion of very low amounts of the ingested amino acids in the urine (2). The question arose whether this observed wastage of amino acids was characteristic of deficiencies of essential amino acids or whether it was a more general phenomenon associated with weight losses due to deficiencies in other dietary essentials. In the present study diets were fed deficient in thiamine, riboflavin, pyridoxine, niacin, calories, total protein, or magnesium, and the urinary excretion of 7 representative amino acids was determined microbiologically.

METHODS

The basal diet fed had the following composition:

	<i>per cent</i>
Casein (extracted)	10
Corn oil (+0.1% halibut liver oil)	5
Wesson's salt mixture (3)	4
Glucose monohydrate (cerelose) to	100

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	γ/μ
Pyridoxine hydrochloride	6
Thiamine chloride	6
Nicotinic acid	10
Calcium pantothenate	20
Riboflavin	6
Folic acid	0.5
Biotin	0.5
<i>p</i> -Aminobenzoic acid	300
Inositol	500
Choline chloride	1000

In specific diets the thiamine, riboflavin, niacin, or magnesium was omitted from this mixture; in the low protein diet, the casein was replaced by glucose. Diets devoid of pyridoxine contained either 10% or 50% of casein, since the severity of these deficiency symptoms in the mouse depends upon the percentage of protein in the diet (4). Appropriate control groups were fed similar diets containing 6 mg. of pyridoxine hydrochloride/kg. of diet. The low calorie groups were fed the control diet restricted in amount equivalent to that consumed by the group deficient in thiamine.

Most of the diets were fed to groups of 6 young adult albino mice or to weanlings 9–12 g. in weight. The deficient diets were fed for at least 7 days prior to the collection of the urine, and usually for much longer periods of time, the time of depletion depending upon the specific deficiency involved (see tables). Thus, certain groups deficient in riboflavin or magnesium were depleted for as long as 55 days prior to the collection of urine. Groups of 4 mice were then placed in metabolism cages, and the urine collected for periods of 24–72 hr. and the samples diluted, preserved, and analyzed microbiologically as in previous studies (5). Determinations were made on both hydrolyzed (2) and unhydrolyzed samples of urine.

EXPERIMENTAL

Thiamine Deficiency, Caloric Restriction or Absence of Protein

Adult mice severely deficient in thiamine and losing 0.57 g./day in weight after 10 days of depletion excreted less "free" aspartic acid, glycine, glutamic acid, histidine, phenylalanine, proline, and valine daily (Table I) than did control mice receiving 6 γ of thiamine/g. of diet.² The differences in excretion, however, seemed to be due primarily to the low intake of food by the thiamine-deficient mice; and the percentages of the ingested amino acids appearing in the urine, a mean of 3.58% of free amino acids, were higher in the thiamine-deficient group than in the control group, which excreted a mean of 1.41% of the ingested amino acids in the free form. The excretion of "total" amino acids by the two groups, however, was more nearly equal. The percentages of ingested amino acids excreted on the diet deficient in thiamine fell within the comparatively narrow range of 4.19–5.68% for six of the seven amino acids determined, while the percentage of ingested glycine excreted was 33.6%. Normal mice² excreted 31.5% of the ingested glycine. The amounts of glycine excreted, however, were not

² Protocols of these experiments appear in a thesis entitled "Metabolism of Amino Acids by Normal and Deficient Rats and Mice" H. E. Sauberlich, University of Wisconsin, 1948. The data are summarized in Table V of the present paper.

TABLE I

Urinary Excretion of Amino Acids by Adult Mice on Diets Deficient in Thiamine or in Calories

Amino acid		10% Casein—no thiamine			10% Casein—low calories		
		Amino acid ingested	Amino acid excreted	Percent-age excreted	Amino acid ingested	Amino acid excreted	Percent-age excreted
Valine	"Free"	mg. 8.74	mg. 0.35	4.0	mg. 9.49	mg. 0.19	1.9
	Total		0.41	4.6		0.20	2.0
Phenylalanine	"Free"	7.19	0.27	3.8	7.80	0.11	1.4
	Total		0.41	5.6		0.25	3.1
Glutamic acid	"Free"	30.31	1.05	3.4	32.89	0.42	1.2
	Total		1.69	5.5		0.99	3.0
Histidine	"Free"	4.23	0.11	2.5	4.59	0.06	1.3
	Total		0.18	4.1		0.13	2.8
Aspartic acid	"Free"	10.29	0.26	2.5	11.17	0.27	2.4
	Total		0.54	5.2		0.75	6.7
Proline	"Free"	18.47	0.66	3.5	20.04	0.24	1.2
	Total		0.84	5.0		0.37	1.8
Glycine	"Free"	2.52	0.47	18.4	2.76	0.50	18.2
	Total		0.85	33.6		1.32	47.9

Diet	Initial wt.	At time of collection			
		Time	Ave. wt.	Wt. change per day	Daily food intake
10% Casein—thiamine	g. 26.6	days 10	g. 16.9	g. -0.57	g. 1.41
10% Casein—restricted (to thiamine-deficient mice)	26.0	9	21.0	-0.45	1.53

very different from those of other amino acids and were actually less than the amounts of glutamic acid found in the urine. The relatively high percentages of ingested glycine excreted, therefore, were attributed to the facts that the intake of glycine was low and that glycine can arise from other substances in the body (6).

In contrast to the results with adult animals, young mice deficient in thiamine excreted much higher percentages of the ingested amino acids into the urine than normal mice. The mean excretion for 7 amino acids was 20.7% as contrasted to a mean of 4.9% for similar animals receiving adequate amounts of thiamine (Table II).

This has been confirmed in a second series in which 5 additional amino acids were determined. Thus, thiamine deficiency caused a much greater increase in amino acid excretion in young mice than in older animals of this species, in spite of the fact that weight losses in the two groups were equivalent.

TABLE II
Urinary Excretion of Amino Acids by Thiamine-Deficient Mice

Amino acid	10% Casein + thiamine			10% Casein - thiamine		
	Amino acid ingested	Amino acid excreted	Percentage excreted	Amino acid ingested	Amino acid excreted	Percentage excreted
	mg.	mg.		mg.	mg.	
Tyrosine	17.52	0.83	4.7	4.52	0.89	19.7
Glutamic acid	72.45	2.42	3.3	18.70	2.30	12.3
Histidine	10.17	0.47	4.6	2.61	0.51	19.4
Tryptophan	4.72	0.27	5.7	1.22	0.25	20.7
Lysine	25.61	1.27	4.9	6.61	1.43	21.6
Valine	20.89	1.27	6.0	5.39	1.45	26.7
Phenylalanine	17.19	1.16	6.7	4.44	1.09	24.6

Diet	Initial weight	At time of collection			
		Time	Ave. wt.	Wt. change per day	Daily food intake
	g.	days	g.	g.	g.
10% Casein + thiamine	19.3	14	22.9	+0.30	3.37
10% Casein - thiamine	19.0	14	16.7	-0.60	0.87

Adult mice fed the complete diet but restricted in amount to that consumed by the thiamine-deficient group excreted only about half as much of the amino acids as the latter. Of the 7 amino acids determined, the mean excretion by the low calorie group was 0.24 mg. daily of the free form and 0.37 mg. of total amino acids (Table I), as compared to 0.35 mg. and 0.54 mg., respectively, by the thiamine-deficient mice of similar age. Calculated as the percentages of ingested amino acids appearing in the urine, the mean amounts excreted in the free form were 1.41% and 3.58% on the two diets, respectively. The mean amounts determined after hydrolysis were 3.02% of the amount ingested by the low calorie group as compared with 5.22% for the thiamine-deficient animals. Thus, in mice losing weight due to a low caloric intake, the excretion of amino acids was minimized rather than enhanced; thiamine-deficient mice, on the other hand, excreted more amino acids than control mice receiving equivalent amounts of calories and protein.

Adult mice fed a diet devoid of protein consumed 3.68 g. of food daily as compared to 4.0-4.2 g. by mice receiving 10% of casein. The protein-deficient mice lost 0.05 g. in weight per day after 7 days on the deficient diet, and excreted very low amounts of

the various amino acids in the urine, 0.054 to 0.606 mg./day for 6 of the 7 amino acids determined. Unexpectedly, however, the excretion of glycine by the protein-deficient mice was as high as that observed on any of the normal or deficient diets studied, *viz.*, 1.97 mg./day in the free form and 2.95 mg./day observed after hydrolysis.

TABLE III
Urinary Excretion of Amino Acids by Pyridoxine-Deficient Mice

Amino acid		50% Casein + pyridoxine			50% Casein - pyridoxine		
		Amino acid ingested	Amino acid excreted	Percent-age excreted	Amino acid ingested	Amino acid excreted	Percent-age excreted
Valine	"Free"	mg. 56.73	mg. 3.02	5.3	mg. 44.02	mg. 4.17	9.4
	Total		3.26	5.7		4.68	10.6
Phenylalanine	"Free"	44.66	2.37	5.0	36.21	2.61	7.2
	Total		2.76	5.9		2.63	7.2
Glutamic acid	"Free"	196.72	6.83	3.4	152.65	9.24	6.0
	Total		12.62	6.4		14.98	9.7
Histidine	"Free"	27.45	1.09	3.9	21.30	1.36	6.3
	Total		1.33	4.8		2.09	9.8
Aspartic acid	"Free"	66.80	2.38	3.5	51.83	2.82	5.4
	Total		4.88	7.2		5.25	10.1
Proline	"Free"	119.86	4.42	3.6	93.01	6.16	6.6
	Total		6.25	5.2		7.96	8.5
Glycine	"Free"	16.47	1.79	10.9	12.78	1.40	11.0
	Total		3.08	18.7		1.82	14.3

Diet	Initial weight	At time of collection			
		Time	Ave. wt.	Wt. change per day	Daily food intake
50% Casein + pyridoxine	g. 10.9	days 12	g. 16.1	g. -0.03	g. 1.83
50% Casein - pyridoxine	g. 9.4	days 12	g. 10.6	g. -0.33	g. 1.42

Pyridoxine Deficiency

Weanling mice severely deficient in pyridoxine excreted somewhat larger amounts of amino acids in the urine than comparable mice fed diets containing 6 γ of pyridoxine/g. (Table III). The results were somewhat similar whether the mice received

10% or 50% of casein in the diet, although the mice on the higher level of protein appeared to be in a more severe state of pyridoxine-deficiency (4) and lost weight more rapidly, -0.10 vs. -0.33 g./day after 2 weeks. The percentages of ingested amino acids excreted by the deficient mice on the high protein diet were somewhat greater than those on the lower level of casein, a mean of 9.8% as compared to 6.6%. Comparable animals receiving pyridoxine excreted less of most of the amino acids than the deficient animals. The daily mean excretion of free and total amino acids was 2.82 mg. and 4.68 mg., respectively, for the pyridoxine-deficient mice receiving 50% of casein as compared to a mean excretion of 2.38 mg. and 3.26 mg., respectively, for

TABLE IV
*Urinary Excretion of Amino Acids by Magnesium-
and Riboflavin-Deficient Mice*

Amino acid		10% Casein—riboflavin			10% Casein—magnesium		
		Amino acid ingested	Amino acid excreted	Percentage excreted	Amino acid ingested	Amino acid excreted	Percentage excreted
Valine	"Free"	mg. 8.37	mg. 0.39	4.7	mg. 11.28	mg. 0.86	7.6
	Total		0.46	5.4		1.13	10.0
Phenylalanine	"Free"	6.88	0.26	3.7	9.28	0.77	8.3
	Total		0.32	4.6		1.12	12.0
Glutamic acid	"Free"	29.02	0.67	2.3	38.13	2.19	5.6
	Total		1.11	3.8		3.89	9.9
Histidine	"Free"	4.05	0.12	2.9	5.46	0.35	6.3
	Total		0.16	3.9		0.40	7.2
Aspartic acid	"Free"	9.85	0.27	2.7	13.29	0.59	4.4
	Total		0.50	5.0		1.34	10.0
Proline	"Free"	17.68	0.39	2.1	23.84	1.43	5.9
	Total		0.47	2.6		2.07	8.6
Glycine	"Free"	2.43	0.63	25.9	3.28	0.64	19.5
	Total		0.04	42.9		0.90	27.5

Diet	Initial weight	At time of collection			
		Time	Ave. wt.	Wt. change per day	Daily food intake
10% Casein—riboflavin	g. 10.0	days 25	g. 11.6	g. -0.40	g. 1.35
10% Casein—magnesium	g. 9.3	days 55	g. 16.4	g. -0.08	g. 1.82

comparable mice receiving adequate amounts of pyridoxine (Table III). The mean percentages of the ingested amino acids appearing in the urine were 9.82% by the deficient group as compared to 5.93% by those receiving pyridoxine (Table III).

The results, therefore, indicated that the excretion of amino acids is increased slightly in pyridoxine-deficiency and that the extent of this excretion tends to parallel the degree of deficiency. Quantitatively, however, the increased loss of amino acids by the young pyridoxine-deficient mouse was much less than that by the young thiamine-deficient mouse (Table II) or by young mice deficient in methionine or tryptophan (1).

Deficiency of Magnesium, Riboflavin, or Nicotinic Acid

Young mice fed a diet deficient in magnesium grew for several weeks, but after 55 days they were losing weight at a rate of 0.08 g./day. In this deficiency, amino acid excretion was increased moderately for 6 of the 7 amino acids determined, glycine being the exception. The mean percentage excretion was 6.35% (Table IV) as compared to a mean of 3.7% in control animals (Table V). The excretion of total amino

TABLE V
Comparative Effects of Various Deficiencies upon Amino Acid Excretion by Mice

Deficiency	Time of depletion	Weight change at time of collection	Daily food intake	Mean excretion for 7 amino acids	
				"Free"	Total
	<i>days</i>	<i>g.</i>	<i>g.</i>	<i>per cent</i>	<i>per cent</i>
None: 10% casein, young	16	+0.20	3.31	3.7	4.5
None: 10% casein, adult	10	+0.30	4.20	1.4	5.3
None: 10% casein, adult	18	0.00	3.87	1.5	5.0
None: 50% casein, young	12	-0.03	1.83	3.7	5.9
None: 9% casein, 6% gelatin	12	+0.20	3.31	2.6	3.8
No protein, adult	7	-0.05	3.68	— ^a	— ^a
Caloric restricted, adult	9	-0.45	1.53	1.4	3.0
Caloric restricted, adult	17	-0.19	1.25	0.7	1.9
Thiamine-deficient, young	14	-0.60	0.87	20.7	—
Thiamine-deficient, adult	10	-0.57	1.41	3.6	5.2
Riboflavin-deficient, young	25	-0.40	1.35	2.9	5.6
Riboflavin-deficient, adult	14	-0.36	3.15	3.5	—
Magnesium-deficient, young	55	-0.08	1.82	6.3	10.0
Pyridoxine-deficient, young					
10% casein diet	16	-0.10	2.08	5.3	6.6
50% casein diet	12	-0.33	1.42	6.4	9.8
Niacin-deficient, young	16	+0.26	2.62	1.9	3.2
Methionine-deficient, young (1)	15	-0.22	1.90	30.4	—
Tryptophan-deficient, young (1)	15	-0.20	1.70	35.0	—

^a Total amount excreted was less than 20% of that on a control diet containing 10% of casein.

acids in the urine was also increased moderately by the magnesium-deficient mice. By way of contrast, young riboflavin-deficient mice did not excrete any more amino acids than control animals receiving riboflavin, in spite of the fact that they were losing appreciable amounts of weight as a result of the deficiency, 0.40 g./day (Table IV). This was confirmed in another series with adult mice.

The contrasting results in the deficiency of riboflavin and thiamine, therefore, suggest that weight loss is not the primary factor determining an excess excretion of amino acids by mice. Attempts to increase the excretion of amino acids by feeding diets deficient in nicotinic acid or in which a relatively high amount of gelatin was present did not result in any increased excretion of amino acids (Table V). The total amount of glycine excreted on the latter diet was high, in line with the high amount of glycine ingested in the form of gelatin, but the percentage excretion of glycine was actually lower on the high glycine diet than when a diet low in glycine was fed. 12.1% total vs. 31.5%. This suggests that the synthesis of glycine from other substances is apparently minimized when a high amount of glycine from exogenous sources is present in the body.

DISCUSSION

Relative Excretion of Amino Acids on Various Deficiencies

In Table V relevant data from the appropriate experiments are summarized to permit a comparison of the excretion of amino acids under various deficiencies and degrees of weight loss. A mean excretion in the "free form" of 4% of the ingested amino acids is arbitrarily regarded as normal, and values higher than this are considered as indicating an increased excretion of amino acids. Similarly, a mean excretion of 6% of the ingested amino acids detected in the urine after hydrolysis is regarded as normal (Table V, lines 1-4), and values greater than this as indicative of some wastage of amino acids as a result of the deficiency. According to these criteria, no increased excretion of amino acids occurred in mice deficient in calories, or riboflavin, or in adult mice depleted of thiamine, in spite of the fact that all of these groups were losing appreciable amounts of weight. As a matter of fact, the group restricted in calories excreted a somewhat lower percentage of the ingested amino acids in the urine than normal mice.

Pyridoxine-deficient mice excreted amino acids in amounts only slightly above those excreted by normal animals, although, when the deficiency was aggravated by a high protein intake, the excretion of amino acids was increased moderately. A moderate increase in amino acid excretion was also observed in magnesium deficiency, although the animals in this latter state were not losing weight rapidly, a further indication that the excretion of amino acids does not necessarily parallel

changes in weight. In the present study, a marked increase in amino acid excretion was observed only in thiamine-deficient young mice. This was confirmed in 2 separate series performed at intervals of 6 months. The mean excretion of amino acids in the free form in one series was 20.7% (Table V) and 19.5% in the second series. By way of comparison, the mean excretion of 15 amino acids by mice fed diets deficient in tryptophan or methionone was 35.0% and 30.4%, respectively, in a previous study (1). Apparently, therefore, these latter spectacular losses of amino acids are in part characteristic of deficiencies in the essential amino acids and cannot be ascribed solely to the loss in weight by mice in this condition.

Schweigert (7) has observed that rats deficient in nicotinic acid did not excrete any increased amounts of amino acids in the urine. However, rats fed a riboflavin-deficient diet excreted approximately twice as much of the ingested amino acids in the urine as animals fed an adequately supplemented diet. Similar experiments have been performed in this laboratory on rats deficient in pyridoxine, riboflavin, thiamine, calories, and protein, with only slight increases in amino acid excretion being observed in the groups deficient in riboflavin or pyridoxine (50% casein). Rats deficient in tryptophan or methionine excrete about twice as much amino acid as normal rats (2,7).

SUMMARY

1. Mice were fed diets deficient in protein, calories, thiamine, riboflavin, pyridoxine, or nicotinic acid for varying periods of time until the animals lost weight appreciably.
2. Mice on the protein-deficient diet excreted very low amounts of the various amino acids. Mice fed diets deficient in calories excreted lower percentages of the amino acids ingested than normal mice.
3. Mice fed diets deficient in riboflavin, nicotinic acid, or adult mice deficient in thiamine excreted essentially the same amounts and percentages of the ingested amino acids as normal mice, in spite of significant losses in weight by the animals. Mice fed diets deficient in magnesium or in pyridoxine excreted somewhat higher percentages of the ingested amino acids than normal mice.
4. The greatest losses of amino acids in the urine were observed in groups of young mice fed a thiamine-deficient diet. Approximately 20% of the amino acids ingested by such mice appeared in the urine in a

microbiologically available form. This percentage, however, was less than that previously observed in mice deficient in tryptophan or methionine.

5. Fairly high amounts of glycine were excreted both by normal mice, and by mice on the various deficient diets. Glycine excretion was particularly high on a protein-free diet. The amount of glycine excreted was increased somewhat when a diet high in gelatin was fed, but the percentage of ingested glycine excreted was decreased appreciably on the latter diet.

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The Relationship between pH and Fluorescence of Several Organic Compounds

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INTRODUCTION

Many organic compounds can be excited to fluorescence in extremely dilute solutions, and this property has been used to measure very small amounts of a few compounds. Reasonably specific methods are available for determining such biologically important substances as atabrine, chlorophyll a, certain carcinogenic hydrocarbons, nicotinamide and its derivatives and metabolic products, porphyrins, riboflavin, and thiamine. Although numerous quantitative procedures have been devised, little use has been made of fluorescence in identifying organic compounds. The fluorescence of compounds is of such general occurrence that it is, by itself, of slight value in identifying substances, but fluorescence as a function of controllable factors, especially pH, can be as characteristic as absorption spectra and ionization constants. The pH-fluorescence curve is a complex function of the influence of acidity upon the absorption spectrum, upon fluorescent efficiency, and upon other factors. Fink and Hoerbuerger (4,5) measured the intensity of the red fluorescent light from several porphyrins at different acidities and plotted the pH-fluorescence curves. They considered their pH-fluorescence curves to be at least as useful in identifying porphyrins as melting points, elementary analyses, and absorption spectra. Huff and Perlzweig (7) determined the pH-fluorescence curves of synthetic and urinary 4-pyridoxic acid and its lactone, and used the identity of the curves to support their conclusion that the synthetic and urinary pyridoxic acids were the same.

We have used pH-fluorescence curves and fluorescent color to show that the fluorescent substances extracted from oat roots cannot be

identified with a number of naturally-occurring organic substances (6). The crude extracts were separated into 3 fractions, in none of which thiochrome, riboflavin, lumichrome, lumiflavin, the acetone or butanol derivatives of N^1 -methylnicotinamide, 4-pyridoxic acid lactone, porphyrins, or pteridines derived from folic acid, predominated. The pH-fluorescence curves of the above-mentioned substances and others were determined, and the lack of correspondence between them and the curves obtained with 3 fractions isolated from the roots was taken to indicate lack of chemical identity. The fluorescences of the known and unknown substances were measured in the same buffers.

FLUORESCENCE OF KNOWN SUBSTANCES

The substances investigated were used as received without further purification unless otherwise noted. They were dissolved in water, dilute acid, or dilute alkali, and neutralized after diluting to a concentration usually less than 10 γ /ml. Then 1.00 ml. of the stock solution was put into a cylindrical cuvette and diluted to the 5 ml. mark with buffer. The final concentration was chosen so that the maximum fluorescence gave a scale reading of about 100 on the fluorimeter. The Klett fluorimeter¹ was set to about 100 with a 1 mg./l. quinine sulfate standard in a cylindrical cuvette using a Corning 5970 lamp filter and a 3389 photocell filter. The concentration of the known substances, lamp and photocell filters, maximum fluorescence (for quinine standard = 100), pH of maximum fluorescence, and color of the fluorescent light are given in Table I.

The buffers were: 1 *N* hydrochloric acid (pH 0), 0.3 *N* sulfuric acid (pH 1.2), 0.1 *M* citric acid plus 2% of 2 *N* sulfuric acid (pH 2.0-2.1), McIlwain citrate-phosphate buffers (pH 2.7-8.2), mixtures of 0.2 *M* Na_2HPO_4 and 0.2 *M* Na_2CO_3 (pH 9.2-10.3), 0.2 *M* Na_2CO_3 (pH 11.3), 0.01 *N* NaOH (pH 12.1), 0.1 *N* NaOH (pH 13.1), and 1 *N* NaOH (pH 14).

Possible quenching of the fluorescence by the various buffer salts should be considered. The decrease in fluorescence of a substance caused by an added solute or gas that does not absorb any of the exciting light is called quenching. The effectiveness of a given quencher varies with the properties of the fluorescent substance. For example, chloride ion is very active in quenching the fluorescence of quinine but has little effect upon the fluorescence of riboflavin. Kuhn and Moruzzi (8) showed that riboflavin and lumiflavin were quenched to the extent of 7-10% by McIlwain's buffers, glycine-sodium hydroxide, and sodium phosphate buffers as compared with hydrochloric acid-acetate buffer. Kuhn and Vetter (9) observed 2-10% quenching of thiochrome in glycine-sodium hydroxide buffer as compared with phosphate-citrate buffer. It is quite obvious that the quenching is different for each substance and buffer combination. The best procedure would be to measure the fluorescence in solutions of minimum content of salts chosen to show the least quenching. However, as long as the

¹ The junior author is indebted to the Rumford Committee of the American Academy of Arts and Sciences for a grant to cover the purchase of this instrument.

TABLE I

Maximum Fluorescence and the pH at Which it Occurs for 13 Different Compounds

The lamp filter was a Corning 5970; the photocell filters were Corning 3389 and 3060, except where otherwise noted. The fluorescence is given as a potentiometer scale reading at the concentration and pH indicated. The fluorimeter scale was set to 100 with a quinine sulfate standard of 1 mg./l., using the above-mentioned set of filters.

Substance	Source	Mol. wt.	pH	Maximum fluorescence	
				1 mg./l.	1 μ mole/l
Bis-iminoalloxazine	^a	235	3.6	205	48
Lumichrome	^b	242	8.3	112	27
Lumiflavin	^c	256	7.3	178	46
Riboflavin	^{b, c}	376	6.4	187	70
Kühling compound	^d	391	4.5	26	10
Derivatives of 2-amino-4-hydroxy-pteridine:					
6-methyl	^e	177	10.1	420	74
7-methyl	^e	177	9.1	367	65
6-methanol	^a	193	9.1	280	54
6-carboxylic acid	^f	207	9.1	205	43
Anthranilic acid	^{b, g}	137	3.57	4.1	0.55
4-Pyridoxic acid lactone	^h	167	9	150 ^m	25
Quinine sulfate	ⁱ	778	1 ^j	100	77.8
Thiochrome	^c	262	11 ^k	260 ^m	66

^a Dr. G. H. Hitchings.

^b Prepared or purified by the authors.

^c Merck & Co.

^d Hoffman-La Roche, Inc.

^e Lederle Laboratories.

^f Parke-Davis & Co.

^g Eastman Kodak Co.

^h Dr. W. A. Perlzweig.

ⁱ J. T. Baker & Co.

^j Quinine measurement at pH 1. The fluorescence of quinine increases with increasing concentrations of sulfuric acid.

^k Thiochrome measurement at pH 11. The fluorescence may be slightly greater in more alkaline solutions.

^m Thiamine photocell filter (Corning 3389 + 4303.)

same buffers are used for the known and unknown substances, quenching by the buffer salts will not interfere with the identifications. Weil-Malherbe and Weiss (13) have shown that quenching by dissolved oxygen can be a large and disturbing factor in the measurement of fluorescence, especially the fluorescence of carcinogenic hydrocarbons. Unless quenching by oxygen is very large, it is more convenient to obtain maximum

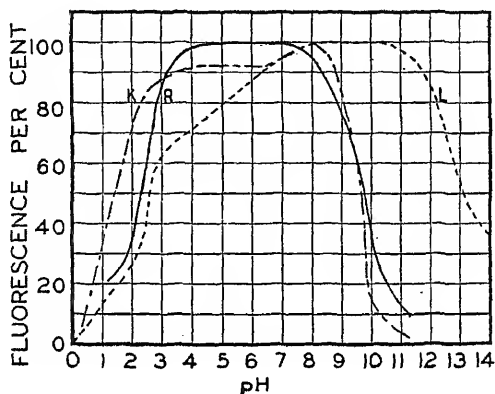


FIG. 1. The pH-fluorescence curves of riboflavin and related compounds. Riboflavin (R), —; lumichrome (L), ---; the Kühling compound (K), ——. The curve for lumiflavin is nearly identical with that of riboflavin.

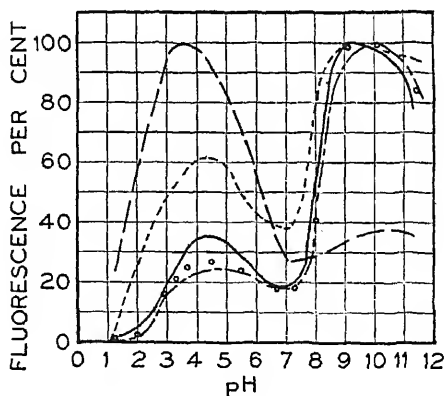


FIG. 2. The pH-fluorescence curves of *bis-iminoalloxazine*, —; and of derivatives of 2-amino-4-hydroxy-pteridine: 6-methanol, ---; 6-carboxylic acid, ——. The curve for the 7-methyl derivative is so similar to that of the 6-methyl derivative that the data have been shown as circles and the curve has not been drawn.

quenching by saturating the solution with air than to remove all dissolved oxygen. When the unquenched values of fluorescence must be known, as in the determination of ionization constants from the shape of the pH-fluorescence curve, possible quenching by the buffers and by oxygen should be considered.

The curves obtained by plotting relative fluorescence for each substance are more easily compared than are the curves obtained by plotting the fluorescence (potentiometer scale readings) against pH. The relative fluorescences given in Figs. 1, 2, and 3 were computed by dividing the fluorescence at each pH by the maximum fluorescence and multiplying by 100.

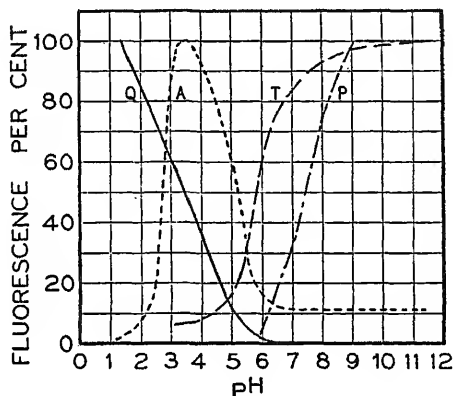


FIG. 3. The pH-fluorescence curves of several miscellaneous compounds. Quinine (Q), —; thiochrome (T), — — —; anthranilic acid (A), - - - -; 4-pyridoxic acid lactone (P), — · — ·.

The few published pH-fluorescence curves resulting from measurements with modern instruments are in good agreement with those given here. Among the measurements that should be mentioned are those made by Ellinger and Holden (3) on riboflavin and thiochrome with an instrument similar to ours, and the visual measurements of Kuhn and Moruzzi (8) on riboflavin, Kuhn and Vetter (9) on thiochrome, and Desha, Sherrill and Harrison (2) on quinine sulfate. The curve for 4-pyridoxic acid lactone does not agree very well with that of Huff and Perlzweig (7).

FLAVINS

The general shape of the curves for riboflavin, its two decomposition products—lumiflavin and lumichrome, and the Kühling compound

(Fig. 1) is that expected if the molecule responsible for the emission of light is the nonionized form of an amphoteric substance. The curves for riboflavin and lumiflavin are nearly identical throughout the entire pH range. Since both are 9-substituted derivatives of 6,7-dimethylisoalloxazine, this correspondence of curves is not unexpected. Whether or not the 9 position of the isalloxazine ring is substituted by a methyl or a *D*-ribityl radical makes little difference, but a large shift in each end of the curve results if the 9 position is unsubstituted as it is in lumichrome. The curve for lumichrome (6,7-dimethylalloxazine) is that of a sample prepared from riboflavin, and is nearly the same as the curve obtained from two other samples, one from Merck & Co., and one from Dr. Jackson W. Foster. It is shifted toward the alkaline region by about 0.5 pH unit in acid solution and by about 3 pH units in alkaline solution when compared with riboflavin. The color of the fluorescent light was the same yellow-green for both riboflavin and lumiflavin between pH 1 and pH 10. Above about pH 10, the fluorescent color became progressively bluish with increase in pH. The fluorescent color of lumichrome was bluish-green below and greenish-yellow above pH 4. The photodecomposition of riboflavin was rapid between pH 4 and 8, less rapid at pH 3 and 9, and very slow at pH 2 and 10. The rate of decomposition seems to be related to the concentration of the non-ionized molecule; the greater the concentration, the more rapid the decomposition. At pH 11.3, the measured fluorescence increases with the period of irradiation. Since, at pH 11.3, the fluorescence, on a molecular basis, of lumiflavin is about one-half that of riboflavin, whereas the fluorescence of lumichrome is about 6 times as great, the observed increase in fluorescence probably results from conversion of riboflavin into lumichrome. The formation of lumichrome is slow in the fluorimeter. The Kühling compound, 5-(6-*D*-ribitylamino-3,4-dimethylphenylimino)-barbituric acid anil (11), gave a pH-fluorescence curve with the same general shape as that of riboflavin. Like riboflavin, its photodecomposition was most rapid between pH 3 and 8, and the fluorescent color was bright green.

PTERIDINES

The second group of related compounds in Table I is that of the pteridine derivatives: 2-amino-4-hydroxy-6-methylpteridine, 2-amino-4-hydroxy-pteridine-6-methanol, 2-amino-4-hydroxy-pteridine-6-car-

boxylic acid, and 2-amino-4-hydroxy-7-methylpteridine. The curves for these compounds are given in Fig. 2. Bisiminoalloxazine is also included in Fig. 2, because it has one ring identical with one ring of the pteridines. The shape of the curves is that expected if both the neutral molecule and an anion are fluorescent. The peak at pH 4.5 represents the fluorescence of the neutral molecule.

Folic acid is not included because the pure acid is not fluorescent (10); we have confirmed this observation. The fluorescence of pterioic acid is very weak and could be accounted for by the presence of 1% of 2-amino-4-hydroxy-6-methylpteridine. Thus, pterioic acid, like folic acid, is not fluorescent at pH values between 1.2 and 11.3. There is ample absorption of the 366 $m\mu$ line by both compounds (12), so the absence of fluorescence is not caused by lack of absorption of the exciting line. Pterioic acid solutions adjusted to pH values of 9 or higher and irradiated in the fluorimeter showed a rapid increase of fluorescence, indicating a photodecomposition of the pterioic acid to a pteridine. The pH-fluorescence curve of the pteridine decomposition product was nearly identical with that of 2-amino-4-hydroxy-pteridine-6-carboxylic acid.

Examination of the data in Table I shows that, as the substituent in the 6 position increases in complexity from methyl to carboxyl, the fluorescence decreases, disappearing when the substituent is methylaminobenzoic acid (as in pterioic acid). The 6- and 7-methyl pteridines were equally fluorescent and had nearly identical pH-fluorescence curves.

When only the maximum in the acid range (peak at pH 4.3-4.5) is considered, the pteridines gave curves with the same shape but with quantitative differences, the differences being greater on the acid side than on the alkaline side of the peak. This could indicate differences in the strengths of the basic groups of the molecules by a factor of as much as 10.

The pteroyl heptaglutamic acid was fluorescent and gave a curve that resembled the curve of 2-amino-4-hydroxy-pteridine-6-carboxylic acid more nearly than that of any of the other pteridines. The fluorescence of the pteroyl heptaglutamic acid (B_6 conjugate) is difficult to explain, since pterioic acid and folic acid (*L. casei* fermentation factor, pteroyltriglutamic acid) are not fluorescent. Its fluorescence at pH 9.1 is slightly more than that of an equivalent concentration of the 6-

carboxylic acid derivative or equal to the fluorescence given by two-thirds as much of the 6-methylpteridine, which was the most fluorescent pteridine we measured. In other words, our sample of the B_c conjugate either was entirely decomposed, so that all of its pteridine was free as the 6-carboxylic acid derivative, or it was two-thirds decomposed with the 6-methyl compound as the free pteridine (or less decomposed into some other much more fluorescent pteridine). Another possibility is that the B_c conjugate is naturally fluorescent, but this seems improbable in view of the nonfluorescence of pteric and folic acids. Our sample of the B_c conjugate was not the purest that has been prepared and probably contained contaminating free fluorescent pteridines.

In a recent paper (1), the adsorption of pure "folic acid" on a number of adsorbents was followed fluorometrically. The best adsorbents were then used to remove "folic acid" from tomato juice and liver extract, the adsorption being measured by a microbiological method. The adsorbents that were effective in removing "folic acid" from the pure solution were rather poor or very poor adsorbents of "folic acid" from tomato juice and liver extract. This result is not unexpected, since it was the adsorption of pteridines that was studied by the fluorometric method and the adsorption of "folic acid" by the microbiological method. The pteridines found as contaminants in folic acid preparations and folic acid itself need not have the same adsorbabilities and the work cited above indicates that they do not. This confusion of a fluorescent impurity with the main substances studied results from the extreme sensitivity of the fluorometric method, and emphasizes the necessity of first ascertaining that the fluorescence is not caused by an impurity.

MISCELLANEOUS SUBSTANCES

The third and miscellaneous group of compounds in Table I will now be considered. The pH-fluorescence curves for these compounds are given in Fig. 3. The curve for anthranilic acid indicates that the non-ionized molecule is the main fluorescent species.

The curve for 4-pyridoxic acid lactone suggests that an anion is the fluorescing form.

The curve for quinine sulfate is similar to that of Desha, Sherrill and Harrison (2) obtained by visual methods when their determination at pH 0.85 is taken as 100. The curve is nearly the same as that obtained by plotting the concentration of the doubly charged cation of quinine.

The curve for thiochrome is nearly identical with that of Ellinger and Holden (3), and indicates that an anion is the fluorescent substance. The fluorescent color was blue at pH 5 and above, and yellow at pH 4

and below. The change in the color of the fluorescent light with pH presumably indicates a concomitant change in the type of fluorescing structure. The yellow-fluorescing thiochrome would be the uncharged molecule.

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SUMMARY

The relationship between pH and the intensity and color of the fluorescence of the following organic compounds, lumichrome, lumiflavin, riboflavin, Kühling compound, *bis*-iminoalloxazine, 4 derivatives of 2-amino-4-hydroxy-pteridine, anthranilic acid, 4-pyridoxic acid lactone, quinine sulfate, and thiochrome, has been determined. The shape of the pH-fluorescence curve is similar for the flavin compounds and indicates that the neutral molecule is the fluorescent species. The pH-fluorescence curves for the 4 pteridine derivatives studied show two peaks, one in the acid and one in the alkaline range. Folic acid and pteric acid are not fluorescent; but pteric acid is photolabile at pH values of 9 or above, decomposing into a pteridine (possibly 2-amino-4-hydroxy-pteridine-6-carboxylic acid). The shapes of each of the pH-fluorescence curves of *bis*-iminoalloxazine, anthranilic acid, 4-pyridoxic acid lactone, quinine sulfate, and thiochrome are characteristic. It is pointed out that the shapes of pH-fluorescence curves and the color of the fluorescence are of diagnostic value in the identification of unknown compounds.

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Interaction of Calcium, Phosphorus, and Vitamin D.¹

III. Study of Mode of Action of Vitamin D using Ca⁴⁵

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INTRODUCTION

The mode and site of action of vitamin D still remains a controversial problem. Evidence exists to substantiate the theory that the vitamin influences the absorption of calcium or phosphorus or both (3,5). Experiments with Ca⁴⁵ by Greenberg (2) led to the conclusion that vitamin D has a dual action, that of promoting absorption of calcium and of exerting a direct effect on the mineralization of bone.

Another possible theory of vitamin D action, which explains the observations made on chicks by Migicovsky and Emslie (4), is that the vitamin acts on the newly formed bone, by resisting the solution effect of an adverse mineral equilibrium which exists in a rachitic animal. The promotion of calcium absorption by vitamin D, according to this theory, would be considered a secondary effect.

The feeding experiments of Migicovsky and Emslie (4) have been followed by the present investigation on the mode of action of vitamin D, in which radioactive calcium is used, with excretion of calcium and phosphorus as the criterion of vitamin D activity. The results lead to the conclusion that the initial action of vitamin D is to prevent the solution of bone calcium.

EXPERIMENTAL

One-day old leghorn chicks were fed Assoc. Official Agri. Chemists rachitogenic diet (1) without vitamin D until placed on experiment. After a specified time, chicks were dosed twice with Vitamin D or corn oil, followed by a removal of feed the next day and a repetition of the doses. On the third day the chicks were given another dose of vitamin D and the excreta were collected for 48 hr. Another dose was given during the collection period, on the fourth day.

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With all the experiments, except No. 140, the treatments were given to quadruplicate groups of 10 chicks per group; experiment 140 was carried out in triplicate only.

In experiments where the effect of calcium or phosphate was being studied the mineral dose was given the day the excreta collection began.

The excreta were collected in either two consecutive 24 hr. samples or one 48 hr. sample. The collection was made in galvanized metal pans, washed into beakers and analyzed for calcium and phosphorus, using perchloric acid digestion followed by permanganate titration in the case of calcium, and in the case of phosphorus by titration of the molybdate. It should be pointed out that the term "excreta" refers to the combined urinary and fecal excrement.

The experiments with radioactive calcium were carried out as described above, except that several doses of radioactive calcium as the chloride were given one week prior to the start of the experiment. In this way chicks were prepared that had a deposit of radioactive mineral in the tissues. The dose of radioactive calcium was approx. $0.2 \mu\text{c}/\text{chick}/\text{day}$ for 3 days. These experiments were also conducted in quadruplicate with 5 chicks per group.

Measurement of radioactivity was made with a nucleometer. The calcium was precipitated on a filter paper pad as the oxalate, the thickness of the precipitate being less than $1.0 \text{ mg. Ca}/\text{cm.}^2$ In this manner, absorption corrections were avoided.

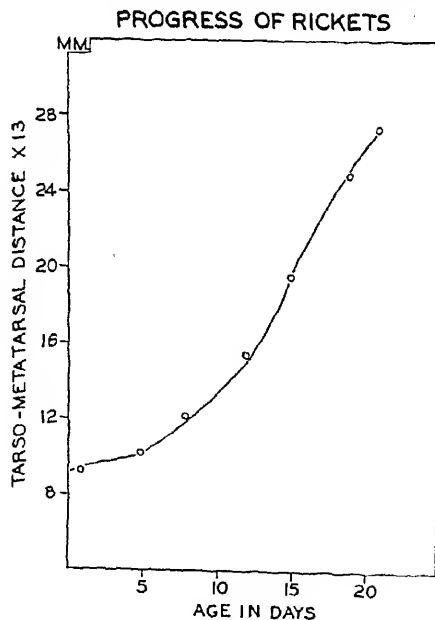


FIG. 1.

The experiments were conducted with one-, two- or three-week old chicks. The one-week old chicks were considered to be non-rachitic. At two weeks the rachitic condition is established. This is substantiated in Fig. 1, where the degree of rickets, as

measured by the tarso-metatarsal distance in an X-ray plate of the joint, is plotted against time.

The results of the first series of experiments are shown in Table I. The data illustrate quite conclusively that, in the non-rachitic one-week old chicks, vitamin D does not exert any influence on the net excretion of calcium or phosphorus. The effect of calcium ingestion on phosphate excretion is quite marked in that it definitely decreases the loss of phosphorus *via* the excreta.

TABLE I
Excretion of Calcium and Phosphorus in Starving Chicks

Experiment no.	Age	Calcium			Necessary diff. at $p = .01$
		Corn oil	Vitamin D	Corn oil and phosphate	
	<i>wk.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	
133	1	46	52	—	18.2
136	1	53	47	53	11.9
137	2	55	33	66	18.5
140	2	62	37	55	26.7
138	3	89	46	84	37.0
Experiment no.	Age	Phosphorus			Necessary diff. at $p = .01$
		Corn oil	Vitamin D	Corn oil and calcium	
	<i>wk.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	
133	1	110	99	47	18.3
136	1	143	136	72	18.2
137	2	207	183	116	34.1
140	2	232	216	161	27.8
138	3	304	244	211	91.0

The data with two- and three-week old chicks, which are rachitic, illustrate that vitamin D decreases the amount of calcium lost in the excreta but exerts very little effect on the phosphorus. The effect of calcium ingestion is the same as with non-rachitic one-week old chicks, namely the loss of phosphate is decreased. Also phosphate ingestion exerts no effect on the excretion of calcium.

If the effect of vitamin D, noted above, is not one of reabsorption, we must conclude that vitamin D influences the retention of calcium and its influence on absorption of calcium is a secondary effect.

To establish whether the decrease in calcium of the excreta is due to retention or reabsorption, the chicks, in which radioactive calcium was deposited, were used.

Since the Ca^{45} was deposited in all the chicks prior to treatment with vitamin D we assume that the specific radioactivity of the calcium in all chicks is the same. If

reabsorption caused the decreased amount of calcium in the excreta the specific radioactivity of the calcium in the excreta would remain unchanged. If retention caused the decrease, the specific radioactivity of the excreta calcium would be less if the retention took place in tissues of higher specific activity and greater if retention took place in tissues of lower specific activity. Therefore, if the specific radioactivity of bone calcium were higher than that for other tissues, and if the specific radioactivity of excreta calcium were less in the vitamin D-treated chicks than in the controls, we must conclude that vitamin D influences the retention of calcium in the bone.

The results of the excretion of Ca^{45} are shown in Table II. It is seen that the specific radioactivity of the excreta calcium from vitamin D-treated chicks is much less than that of the controls.

TABLE II
Effect of Vitamin D on Excretion of Ca^{45}

Expt. no.	Age wk.	mg. Calcium		Nec. diff. at $p = .01$	c.p.m. ^a Ca^{45} /mg. Ca		Nec. diff. at $p = .01$
		Corn oil	Vitamin D		Corn oil	Vitamin D	
144	2	41	26	13	216	102	82
147	2	47	34	20	197	81	73

^a Counts/min.

Analyses of the carcasses of chicks and of the tibiae are shown in Tables III and IV, respectively. It is seen that bone has a higher specific activity than other tissues and also that the activity of newly formed bone, *i.e.*, the epiphyses, is greater than the activity of the diaphyses, thereby leading us to the conclusion that vitamin D exerts a retentive effect on the calcium of newly formed bone.

TABLE III
*Specific Activity (Ca^{45}) of the Chick Carcass
One Week after Dosing with Ca^{45}*

Tissue	c.p.m. ^a /mg. Ca^{45}	
	Corn oil	Vitamin D
Skin	200	250
G. I. tract	123	112
Muscle	254	274
Bone	319	334
Remainder	230	237

^a Counts/min.

Note: Values represent the mean of 4 groups of 2 chicks/group.

Analysis of variance reveals that the variance for tissues is significant at $p = .01$ whereas the variance for treatment is not significant.

TABLE IV
Specific Activity (Ca^{45}) in Epiphyses and Diaphyses of Chick Tibiae

	c.p.m. ^a /mg. Calcium	
	Corn oil	Vitamin D
Epiphyses	555	546
Diaphyses	425	465

^a Counts/min.

Note: The values represent the mean of 4 groups of 10 chicks/group.

The analysis of variance reveals that the variance for tissue is significant at $p = .01$ whereas the variance for treatment is not significant.

Examination of the results in Tables III and IV reveals that there is no difference in specific radioactivity of the calcium in the tissues of vitamin D-treated and negative control chicks. This is the expected result, since the Ca^{45} was deposited prior to vitamin D treatment.

CONCLUSIONS

The results of this investigation on chicks lead us to a different conclusion about the action of vitamin D from that which Greenberg (2) arrived at working with rats. He concluded that vitamin D promotes the absorption of calcium and exerts a direct effect on the mineralization of bone. We conclude that the effect on the mineralization of bone is indirect in that it prevents the solution of bone. Rickets results because bone calcium is being dissolved. This may be prevented by feeding vitamin D or rectifying the level and ratio of calcium and phosphorus in the diet (4). It is a fact that, unless the calcium and phosphorus in the diet are unbalanced, rickets will not result in rats, or, in chicks, only a mild degree of rickets will occur.

We can explain the absorption effect of vitamin D in that, if vitamin D prevents the entrance of bone calcium into the metabolic pool, the tendency is to extract calcium from the gut.

SUMMARY

Experiments on excretion of calcium and phosphorus of starving chicks indicate that vitamin D decreases the loss of calcium *via* the excreta, and has little effect on the phosphorus.

Calcium ingestion decreases the loss of phosphate.

When chicks which were previously fed radioactive calcium were used, vitamin D decreased the specific activity of the excreta calcium, indicating a retention of calcium in a tissue of high specific activity, namely, newly formed bone.

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Polyethenoid Fatty Acid Metabolism. Effect of Dietary Fat on Polyethenoid Fatty Acids of Rat Tissues ¹

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INTRODUCTION

The symptoms developed by rats on a fat-deficient diet have been attributed to the inability of the animal to synthesize linoleic acid (1). The presence of this acid in the organs of deficient animals has been indicated but has not been definitely established because of the inadequate methods available for the determination of the polyenoic acids in animal tissues. Gregory and Drummond (2), using ester fractionation, reported that no linoleic acid was stored in the fat depots of rats on a fat-free diet, but reported considerable linoleic acid in the liver. However, Burr and Brown (3) believed that the results of the English workers were invalid because the symptoms could not be due exclusively to fat deficiency. Banks, Hilditch and Jones (4) reported 2% octadecadienoic acid in the depot fat of their fat-deficient rats.

In 1938, Nunn and Smedley-Maclean (5) reported that the livers of fat-deficient rats contained no arachidonic or higher unsaturated fatty acids detectable as polybromides. Supplementing the diet with linoleic acid resulted in the production of arachidonate, and linolenic acid supplementation resulted in the production of both arachidonate and docosapentaenoate. The same workers later reported the presence

¹ The experimental data are taken from a thesis submitted to the Graduate School of the University of Minnesota by Irma Rieckehoff in partial fulfillment of the requirements for the degree of Master of Science. This work was aided by grants from the Hormel Foundation, the National Livestock and Meat Board, and the National Dairy Council.

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of small amounts of arachidonate in the phospholipides of liver and muscle of fat-deficient rats, but found no arachidonate in neutral fat (6).

Williams and coworkers (7) studied the effect of diet upon lipid content of the rat's organs, but did not characterize the fatty acids. Kahnke (8) applied a modified alkaline conjugation method (9) to the analysis of the fatty acids of the organs of the rat and found considerable quantities of tetraenoic, pentaenoic, and hexaenoic fatty acids present in such organs as heart, liver, and muscle.

Sinclair's classic work showed that the composition of tissue phospholipide is markedly influenced by dietary fat (10). He found (11) that in fat deficiency the phospholipide fatty acids had an iodine number of 100 and neutral fat fatty acids had a value of 60. The iodine number of the phospholipide fatty acids always increased upon supplementation with fat, but the unsaturation of the neutral fat increased or decreased depending upon the fat fed. Sinclair subsequently (12) reported that the ratio of liquid to solid acids in rat phospholipides was constant, regardless of the degree of unsaturation of the mixed acids, indicating that the changes in unsaturation were due to differences in the relative proportions of the unsaturated acids themselves. Ingestion of saturated fat (13) had no influence upon the degree of unsaturation of the phospholipides, and the high degree of unsaturation induced by feeding highly unsaturated fat was maintained despite the subsequent feeding of saturated fat. These observations indicated the high degree of selectivity of phospholipides for highly unsaturated fatty acids.

The present study was begun in an effort to determine the effect of dietary fat upon the polyunsaturated acids of the tissue lipides, using the recently developed spectrophotometric determination of unsaturated fatty acids.

EXPERIMENTAL

The rats used in these studies were adult females which had been on a fat-free diet for about 10 months prior to the beginning of the experiments. The diet consisted of Labco casein 12.0%, sucrose 83%, McCollum's salt mixture No. 185 4.0% and sucrose vitamin B mixture 1.0%. The latter consisted of thiamine 2.0 g., choline 400.0 g., niacin 4.0 g., pyridoxine 1.0 g., calcium pantothenate 5.6 g., 2-methyl-1,4-naphthoquinone 0.08 g., riboflavin 0.75 g., and sucrose 1586.57 g. The rats received 1 drop Viosterol monthly, and twice monthly 1 drop vitamin A mixture consisting of 9 g. lard + 1 g. vitamin A concentrate.

The rats were divided into 3 groups: a control group receiving only the basal diet, a group which received 100 mg. corn oil daily, and a group which received 100 mg. cod liver oil daily. Supplementation was continued for 8 weeks.

At the end of 8 weeks the rats were decapitated and the livers, kidneys, hearts, depot fat, brains, and samples of muscle and skin were removed. The tissues were digested by boiling for 30 min. in 30% aqueous KOH (4 ml./g. tissue). the unsaponifiable fraction extracted with petroleum ether, the aqueous solution acidified, the fatty acids extracted with petroleum ether, and the solvent removed *in vacuo*.

The iodine numbers of the fatty acids were determined by the method of Yasuda (14), and the spectrophotometric determination of the polyenoic acids was made using

the modification of Holman and Burr (9). Approximately 100 mg. fatty acid was used in each sample. A sample of the same cod liver oil was run each time an analysis was made, the cod liver oil acting as a control in lieu of absolute standards for the pentaenoic and hexaenoic acids.

Two rats of each group were decapitated, the blood drained from the bodies, the intestinal tract removed, and the carcass ground in a meat grinder. The lipides were extracted with hot alcohol, the solvent evaporated *in vacuo* and the lipide taken up in petroleum ether. The phospholipide and neutral fat were separated by the acetone-magnesium chloride method, and the fractions saponified. The fatty acids of each fraction were analyzed as described above.

RESULTS AND DISCUSSION

The fatty acid content of the various tissues, the iodine number of the fatty acids, and the extinction coefficients at the wavelengths representing diene, triene, tetraene, pentaene, and hexaene conjugation in the alkaline isomerized tissue fatty acids are all shown in Table I. The double values under each item do not represent duplicate values but are values obtained from 2 separate analyses from 2 groups of rats. The organs of 5 rats were pooled for each sample and analyses were made in duplicate. Because of the lack of hexaenoate and pentaenoate for use as standards, calculation of their concentrations in the tissue fatty acids can be made only on a relative basis. However, an approximation of the contents of diene, triene, and polyene acids can be made assuming that tetraene, pentaene, and hexaene acids develop the same extinction coefficient at 2325, 2700, and 3000 Å as does arachidonate. Table II shows the composition of the various tissue fatty acids with respect to dienoic, trienoic, and polyenoic fatty acids. The occurrence of tetraenoic, pentaenoic, and hexaenoic acids in the tissue fatty acids is shown graphically in Figs. 1, 2, and 3. It should be pointed out that these representations are subject to the error that hexaenoic acid contributes to the absorption at 3000 and 3450 Å, and that pentaenoic acid contributes to the absorption of light at 3000 Å and that the extent of such contribution is not known because pure acids for standards are not available. However, it is apparent that these 3 absorption bands are not due to the same substance, for they vary in proportion from organ to organ.

From an examination of the data presented, several interesting observations are apparent. The presence of polyunsaturated fatty acids in the tissue lipides of fat-deficient rats is demonstrated. However, the content of these fatty acids is far below that of the tissue

TABLE I
Characterization of Rat Tissue Fatty Acids

Tissue	Dietary supplement	Fatty acids <i>per cent</i>	Iodine number	E ₁ ^{1%} cm, 2325 Å	E ₁ ^{1%} cm, 2700 Å	E ₁ ^{1%} cm, 3000 Å	E ₁ ^{1%} cm, 3450 Å	E ₁ ^{1%} cm, 3725 Å
Liver	None	3.53	110	55.3	46.1	14.6	5.8	3.9
	Cod liver oil	3.78	157	39.4	66.7	54.6	50.9	22.7
	Corn oil	3.88	127	131	117	48.2	6.3	1.7
Kidney	None	2.79	81.5	76.5	82.6	22.5	2.75	0.92
	Cod liver oil	2.72	122	57.6	60.4	44.3	27.3	6.04
	Corn oil	2.17	109	89.5	73.4	37.3	4.1	1.15
Heart	None	1.77	115	219	191	35.5	7.0	2.3
	Cod liver oil	2.46	173	198	170	145	110	50.5
	Corn oil	1.24	134	2.34	140	107	18.6	3.72
Brain	None	5.67	95	86.7	75.5	39.9	20.8	11.0
	Cod liver oil	6.15	128	89.0	102	69.4	38.7	23.1
	Corn oil	5.90	114	91.9	81.7	48.3	20.4	9.2
Muscle	None	5.74	63.3	30.7	20.2	4.1	0.97	0.42
	Cod liver oil	3.96	115	31.3	23.8	17.3	13.9	6.2
	Corn oil	5.06	95.6	45.8	9.8	4.3	1.06	0.40
Skin	None		59.5	15.6	7.8	1.7	0.43	0
	Cod liver oil		73.5	24.6	14.4	5.6	3.4	1.4
	Corn oil		70.5	37.8	11.9	5.73	0.22	0.16
Depot fat	None		58.5	15.7	7.8	3.4	0.80	0
	Cod liver oil		75.0	10.6	5.1	1.8	1.4	0.58
	Corn oil		73	35.9	5.98	2.56	1.42	0.74

TABLE II
*Effect of Diet on the Occurrence of the Unsaturated Fatty Acids
 in Tissue Fatty Acids*

Tissue	Dietary supplement	Diene	Triene	Polyene
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Heart	None	5.16	22.2	5.94
	Corn oil	10.51	4.81	18.1
	Cod liver oil	4.34	3.89	24.2
Liver	None	1.40	4.48	2.44
	Corn oil	2.78	9.91	8.06
	Cod liver oil	-2.03	1.80	9.12
Kidney	None	0.40	8.85	3.76
	Corn oil	2.39	5.19	6.23
	Cod liver oil	0.33	2.39	7.40
Brain	None	1.90	5.13	6.66
	Corn oil	1.88	4.84	8.06
	Cod liver oil	-0.25	4.82	11.6
Muscle	None	1.28	2.28	0.69
	Corn oil	3.64	0.79	0.72
	Cod liver oil	1.49	0.97	2.89
Skin	None	0.86	0.86	0.29
	Corn oil	2.63	0.96	0.86
	Cod liver oil	1.91	0.01	0.94
Depot fat	None	0.86	0.63	0.57
	Corn oil	3.0	0.49	0.43
	Cod liver oil	0.60	0.47	0.30

lipides on stock diets as reported by Kahnke (8). It is interesting to note that the polyunsaturated acids are particularly low in the skin and in depot fat. Unfortunately, one still cannot determine conclusively from these data whether there is any linoleic acid in the tissues of the fat-starved rat. It is true that the calculated diene of heart and liver fats is about 5%, but the value is not sufficiently high to exceed the probable error of the method for this component. Again, the lack of pentaene and hexaene standards prevents us from knowing what contribution these make to the absorptions at 2325, 2700, and 3000Å.

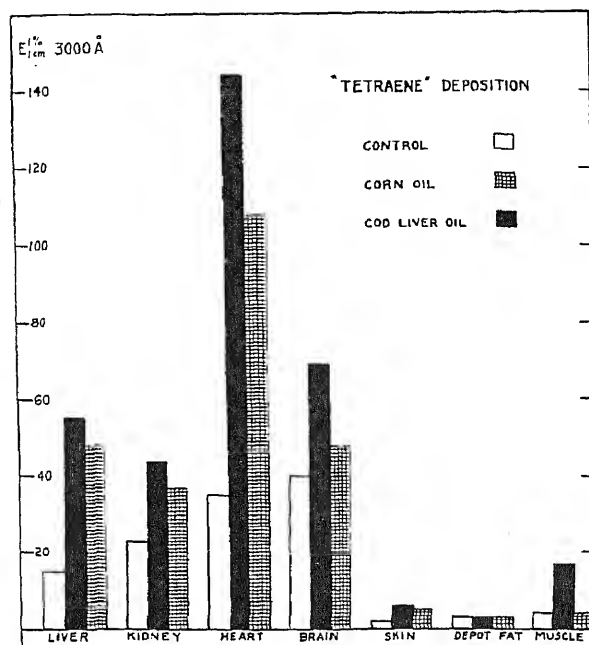


FIG. 1.

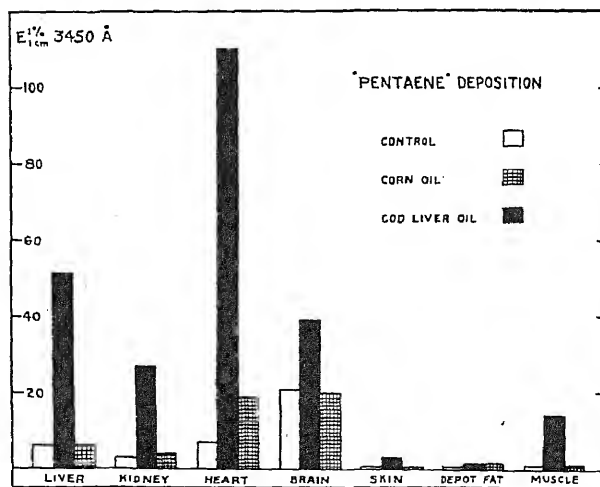


FIG. 2.

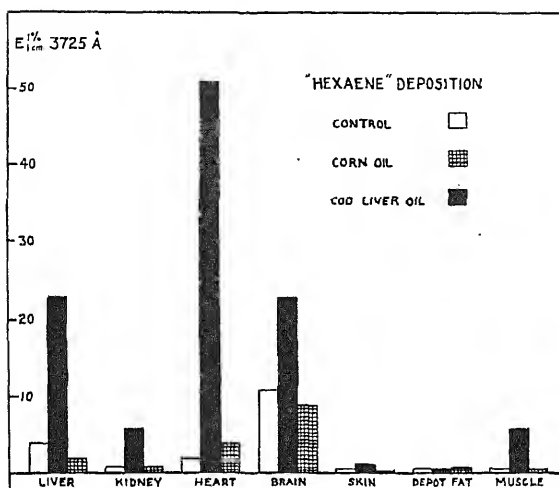


FIG. 3.

The presence of triene in the heart fatty acids in as high a concentration as 22% is surprising, but it should be mentioned that Smedley-Maclean isolated a dihydroarachidonic acid (three double bonds) from the livers of fat-starved rats (5). As she points out, "Apparently in an effort to make the most of the arachidonic acid which had been present when the rat was weaned, the acid had been partially reduced" (15). If we may judge from the observations that (1) the heart retains much of its polyunsaturated acids when the animal is on a fat-free diet and (2) that when these acids are fed to the animal they appear in the heart in the highest proportion, it is natural to postulate that the heart needs these highly unsaturated acids more than the other organs. This may be the explanation for the high percentage of triene in that organ when the animal is on a fat-free diet.

Supplementation with corn oil, which has a high content of linoleic acid, produces some interesting changes in the tissue lipides. No significant deposition of pentaenoic or hexaenoic acids occurred in the tissue lipides except in the case of heart. However, tetraenoic acid (arachidonic acid) contents of the liver, kidney, heart, and brain increased markedly, indicating the likely synthesis of arachidonate from linoleate. No significant changes occurred in the content of the polyunsaturated fatty acid contents of skin, depot fat, or muscle. Corn oil

supplementation strikingly reduced the trienoic acid content of heart fatty acids from 22.2% to 4.8%, a change consistent with Smedley-Maclean's postulate (see Table II). Liver fatty acids exhibit the lone example of an increase in trienoic acid. With the exception of brain fatty acids, all the organ fatty acids increased their content of linoleic, or dienoic acid upon corn oil supplementation. This result would be the expected consequence of feeding an oil high in this acid.

Supplementation with cod liver oil changes the composition of the tissue lipides in quite a different way from that in which supplementation with corn oil does. Most striking is the deposition of pentaenoic and hexaenoic acids in the tissue lipides of liver, kidney, heart, and brain. Deposition of the tetraene, pentaene, and hexaene acids even took place in muscle and skin, which were unaffected by corn oil supplementation. These changes in fatty acid composition with regard to the polyenoic acid undoubtedly reflects a deposition of acids present in cod liver oil rather than synthesis, as was the case with corn oil.

It is significant that the tissues are sensitive to the highly unsaturated acids more or less in the same order in which they retain their highly unsaturated acids when fat-starved for a long time. This suggests that heart, liver, brain, and kidney require highly unsaturated acids, while muscle, skin, and depot fat can function without them when dietary supply is inadequate or when the diet does not provide adequate precursors for synthesis.

TABLE III

Deposition of Cod Liver Oil Pentaenoic Acid in Tissue Fatty Acids

Tissue	$100 \times \frac{\text{Tissue F. A. pentaene}}{\text{C. L. O. pentaene}}$
Heart	95.3
Liver	43.8
Kidney	23.5
Brain	33.4
Muscle	12.0
Skin	2.93
Depot fat	1.22

The heart accumulates a surprisingly high amount of cod liver oil acids (see Table III). In 1933 Madsen, McCay and Maynard (16) reported heart failure in herbivores fed a synthetic diet and cod liver oil as supplement. They also showed that the unsaponifiable fraction of the cod liver oil was not responsible for the heart failure. In view of the

observation that cod liver oil supplement results in an accumulation of polyethenoid acids virtually equal to the content of these in cod liver oil suggests the possibility that the heart failure may have resulted from the accumulation of polyethenoid acids foreign to the animal, displacing unsaturated fatty acids normally present.

TABLE IV
Characterization of Phospholipide and Neutral Fat Fatty Acids of the Rat

Dietary supplement	Fraction	Iodine number	E ₁ ^{1%} 1 cm. 2325 Å	E ₁ ^{1%} 1 cm. 2700 Å	E ₁ ^{1%} 1 cm. 3000 Å	E ₁ ^{1%} 1 cm. 3450 Å	E ₁ ^{1%} 1 cm. 3725 Å
None	Neutral fat	63.0	15.36	9.30	3.42	0.93	0.93
	Phospholipide	101.5	42.0	40.5	17.64	6.28	3.51
Corn oil	Neutral fat	63.5	24.40	6.66	2.70	1.02	1.02
	Phospholipide	107	54.0	42.0	23.55	7.05	3.75
Cod liver oil	Neutral fat	62.5	15.52	6.56	3.84	2.28	1.52
	Phospholipide	125	38.5	37.5	32.25	15.15	9.27

An inspection of Table IV shows that the bulk of the polyethenoid fatty acids are in phospholipide fatty acids and very little is present in neutral fat fatty acids. This was true in all three groups of animals. The increase in arachidonic acid upon supplementing the diet with corn oil is largely in the phospholipide fraction, the "element constant." This is also true of the deposition of polyethenoic acids from cod liver oil. The reciprocal relationship between trienoic and tetraenoic acid is largely in the phospholipide fraction, and bears out the observation of Sinclair that the ratio of solid to liquid fatty acids of phospholipides remains constant despite changes in dietary fat, and strengthens his conclusion that the iodine number change depends upon the substitution of one unsaturated acid for another.

SUMMARY

(1) The tissue fatty acids of the rat retain considerable amounts of the polyunsaturated fatty acids on a fat free diet.

(2) The trienoic acid content of heart fatty acids of rats on a fat-deficient diet is relatively high, and this component decreases upon supplementation with corn oil or cod liver oil.

We found it desirable for blood sugar work to keep mice at a temperature of 80°F. on abundant food and water, and to bleed them after fasting for nearly 5 hr. A similar fasting schedule is recommended in the routine assay of insulin on mice (U. S. P.). Experiments with histamine were performed, in most cases, on mice that were deprived of food for 4 hr. The supply of water continued throughout the experiments.

Histamine diphosphate was injected intraperitoneally and the mice were bled 30–40 min. later.

Since the median lethal dose for sensitized mice was equal to 25 mg. of histamine diphosphate/kg. of body weight in our recent experiments, it represents about 1/100 of the corresponding dose for normal animals.

Even this amount of histamine was too large to be safely injected because it killed sensitized mice very fast, and it was very difficult to withdraw the blood in sufficient amount for analysis before death. In most of our experiments we, therefore, injected the sensitized mice with 5 or 10 mg. of histamine diphosphate/kg. of body weight. Normal mice received higher doses, up to 1000 mg./kg.

EXPERIMENTAL

Altogether, about 100 samples of mouse blood were analyzed. The difficulty in the study of the influence of different factors on glucose blood level in mice consists in the fact that these animals could not be bled repeatedly. We had, therefore, to compare samples of blood obtained from different mice. However, by maintaining the mice under standard conditions we were able to reproduce comparable results.

Data of our analyses are tabulated in the 3 following tables.

TABLE I
Blood Glucose Level in Normal Mice Injected with Histamine
Dose in mg./kg. of histamine injected intraperitoneally in mice

1000 mg.	100 mg. mg.-% glucose	10 mg.
254	272	129
241 ^a	205	187
	165 ^a	202

^a Figures obtained by Somogyi method, all others by Folin-Wu method.

Table I, based on a group of 8 determinations, is meant to show that the blood sugar level of mice, which is normally 150–160 mg.-%,¹ was markedly raised by injection of histamine; similar observations are reported for animals of other species (5). The hyperglycemia in normal mice was especially pronounced after injecting large amounts of histamine, such as 100–1000 mg. histamine/kg. of body weight. Doses smaller than 10 mg./kg. had as a rule no effect on normal mice. In sharp contradis-

¹ This figure is in good agreement with the value of 173.8 mg.-% for the blood sugar content of normal mice as recently described by Hiestand, Hadley and Mercer (*Proc. Soc. Exptl. Biol. Med.* 65, 324 (1947)).

TABLE II
*Blood Glucose Level in Normal Mice, Sensitized Mice, and
 Mice Sensitized and Injected with Histamine*

Mice	mg.-% Glucose			
	Folin Wu	<i>p</i> =	Somogyi	<i>p</i> =
Normal mice	158±7 (10) ^a	<0.001	126±5 (4)	<0.001
Sensitized mice	88±3 (16)		70±4 (8)	
Sensitized mice injected with histamine	64±6 (11)	<0.001	44±5 (7)	<0.01

^a Number of determinations in parentheses.

tion to the reaction of normal mice small doses of histamine produced lowering of the blood sugar in sensitized animals. This is shown in Table II.

This table shows first that one of the effects of sensitization is a marked lowering of the blood sugar to about 55% of the normal value. This hypoglycemia was found to last at least a week.

Furthermore, injection of small doses of histamine into sensitized mice enhanced their original hypoglycemia and produced an additional decrease to 35-40% of the normal blood sugar. The values for *p* given in the table indicate that the differences of the means of the glucose content of the blood are probably not due to chance.

Determinations of the hemoglobin content and the specific gravity of the blood of normal and sensitized mice by the Evelyn method and the copper sulfate method of Phillips, Van Slyke *et al.* (6), respectively, suggested the absence of significant changes in the composition of the blood.

The specific gravity of normal whole mouse blood was found to be 1.056; in sensitized mice the corresponding value was 1.053. The average hemoglobin content of 6 normal mice was 13.85 (min. 11.71; max. 15.79); in the same number of sensitized mice the average hemoglobin content was 12.22 (min. 11.05; max. 13.50). These differences seem hardly significant, thus indicating that general changes of the blood had not occurred and did not interfere with the blood sugar values.

The change in blood sugar level upon injection of histamine inspired us to investigate the role of the pancreas in this phenomenon. Several experiments were carried out along this line, and further work is still in progress. However, some results thus far obtained deserve mention here. To elucidate the difference regarding the endocrine function of the pancreas in normal and sensitized mice, the blood sugar in alloxan-induced diabetes was studied. Waisbren (7) described recently that intravenous injection of 2 mg. alloxan/20 g. mice produced severe diabetes. Our experiments (Table III), in which several groups of normal mice were injected intravenously with 50-100 mg. alloxan/kg., confirmed the findings of this author. Sensitized mice which received the alloxan injection 3-4 days after the sensitizing vaccine injection did not show as

high a blood sugar as the normal mice. The sensitization of mice with vaccine seemed to interfere with the development of diabetes due to injection of alloxan. Although the blood sugar of sensitized mice was increased by alloxan injection it was considerably lower than that of non-sensitized alloxan-treated mice.

TABLE III
*Effect of Alloxan Injection on the Blood Sugar of Normal
and Sensitized (*H. pertussis*) Mice*

Alloxan mg./kg.	Mice	mg. Glucose %			Analytical method
		Ave.	Min.	Max.	
100	Normal	507 (3) ^a	468	536	Folin Wu
	Sensitized	210 (3)	76	350	Folin Wu
	Normal	390 (1)			Somogyi
	Sensitized	146 (1)			Somogyi
50	Normal	217 (2)	146	288	Somogyi
	Sensitized	79 (2)	72	86	Somogyi

^a Figures in parentheses are the number of determinations.

DISCUSSION

The experiments described in this paper bring evidence that the sensitization of mice by administration of a heavy suspension of *H. pertussis* vaccine produced a lasting hypoglycemia, which also interfered with the diabetogenic effect of alloxan. Furthermore, these sensitized mice showed a surprising reaction to histamine. Instead of the hyperglycemia which histamine produced in normal mice, the glucose concentration in the blood exhibited a further drop if small, but not lethal, doses of histamine were given to the sensitized animals.

It is known that bacterial products may lower the blood sugar level of experimental animals (8). Particularly, endotoxins, *e.g.*, of *meningocci* and members of the *salmonella* group, produced in rabbits hyperglycemia, followed by hypoglycemia (Kun and Miller (9)). Kun (10) connected this phenomenon with the influence of endotoxins on carbohydrate metabolism and glycolysis. It seems that the inhibition of glycolysis brought about by certain impurities of penicillin, as described by Miller, Hawk and Boor (11), also might account for the effect of crude penicillin on endotoxin poisoning of mice.

Little is known about the role of histamine in carbohydrate metabolism. According to Billig and Hesser (12), maximal concentrations of histamine were found during the deepest shock in insulin shock treatment, and the injection of glucose relieved not only the shock symptoms, but also reduced the histamine level of the blood. This seems to indicate a connection between insulin activity and histamine effect, although small intramuscular doses of histamine failed to produce an effect on the blood sugar in man, as reported by Hiestand and Hall (13).

Whether the effect of the sensitization by *H. pertussis* vaccine, and the unexpected reaction of histamine on the blood sugar level of mice, is due to a stimulation of the endocrine function of the pancreas, or to an antagonistic influence on the adrenals or the pituitary gland, as suggested by the work of Noble and Collip (14), cannot yet be decided on the basis of our present experience.

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SUMMARY

1. Sensitization of mice with concentrated *H. pertussis* vaccine produced a lasting hypoglycemia.
2. Injection of small amounts of histamine diphosphate into sensitized mice brought about an additional drop of the blood sugar, while normal mice responded to high doses of histamine with hyperglycemia.
3. The hyperglycemia induced by alloxan is significantly lower in sensitized mice than in normal animals treated with the same doses of alloxan.

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A Preliminary Quantitative Study of Pterine Pigment in the Developing Egg of the Grasshopper, *Melanoplus differentialis*¹

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INTRODUCTION

In spite of the greatly increased interest in pterine pigments during recent years, no investigation seems to have given attention to the utilization of these compounds in a developing egg. The occurrence of a white pigment in the wing scales of the butterfly (*Pieris brassicae*) and a yellow pigment in the wings of *Gonepteryx rhamni* have been previously described (1). Crystalline compounds were prepared from these white and yellow pigments and were named leucopterine and xanthopterine respectively (2). An excellent review on the chemistry of pterines is at hand (3).

The quantity of riboflavin in the newly laid egg (*M. differentialis*) remains constant until late in the postdiapause of the embryo (4). In the late postdiapause the acid fluorescence (pH 4.5), due to riboflavin, begins to decrease, and a simultaneous increase of fluorescent alkaline material (pH 10), due to compounds other than riboflavin, occurred (4). Bodine and Fitzgerald (4) suggested that the alkaline fluorescent materials may well be derived from the original riboflavin. The present paper is a report of the results obtained from a study of the possible origins of pterine compounds and their relationship to riboflavin in the egg of the common grasshopper, *Melanoplus differentialis*, during the entire course of its development.

MATERIAL AND METHODS

Grasshopper eggs with known temperature history and known chronological ages, were obtained by methods previously described (5). The shells or chorions were also

¹ Aided by a grant from the National Institute of Health.

removed according to the techniques described (6). The diapause block of the eggs was broken according to the methods previously described (5,8,9).

Eggs in lots of 500 were washed carefully, and those without scars or blemishes were selected for experimentation. The eggs were ground in a glass mortar and extracted successively with small portions of ethyl ether until the ether layer was finally colorless. This method was suitable for the removal of all fatty constituents. The egg brei was heated for 10 min. over a water bath with just enough 95% ethanol to cover the mass (7). The alcoholic solution was separated from the brei, while still warm, by centrifugation. The brei was then extracted with just enough hot water to cover the mass until the final extract became colorless. The alcoholic and aqueous extracts were left at 5°C. for 24 hr. A light brown precipitate occurred upon chilling the solutions.

The light brown residue of the alcoholic solution was extracted three times with cold 0.1 *N* HCl. Within 24 hr., a light brown crystalline compound settled at the bottom of the acid solution. This compound was recrystallized several times from hot 0.1 *N* HCl. The residue from the original acid extraction was treated with a hot solution of 0.1 *N* HCl and centrifuged while hot. As a result of this treatment a small quantity of dark brown amorphous residue settled to the bottom of the golden yellow solution. After 3 hot acid extractions, the amorphous residue was finally discarded. Upon cooling, there was precipitated from the golden yellow acid solution, a small quantity of purplish-brown disc-shaped crystals. The resulting precipitate was washed 3 times with cold water.

The original golden aqueous extracts were combined and the dark brown amorphous residue was discarded. The aqueous extract was made slightly acid with 0.1 *N* HCl and transferred quantitatively to an evaporating dish and left in a desiccator under reduced pressure. When the extract was reduced to $\frac{2}{3}$ of its original volume, a light brown residue deposited on the side of the vessel. The residue was extracted 3 times with a hot solution of 0.1 *N* HCl. The insoluble residue was discarded. Upon cooling, a light brown crystalline compound precipitated out and settled at the bottom of the acid solution. The resulting precipitate was washed 3 times with cold water. The pigmented compound crystallizes into disc-shaped pellets with numerous furrows or rays. When the remaining golden yellow extract was reduced to $\frac{1}{3}$ of its original value, a light brown residue occurred on the side of the vessel and a purplish-brown precipitate settled out on the bottom. The latter compound was the same purplish-brown pigment previously described. The light brown crystalline compound was identical with the pigment previously described as having disc-shaped pellets with numerous furrows. These precipitates were washed 3 times with cold water and dried in a desiccator over concentrated H_2SO_4 . The remaining portion of the aqueous extract was reduced to dryness, yielding a dark amorphous substance. The amorphous substance was finally discarded after several attempts to crystallize it failed. Three distinctly different pterines were isolated by the use of this method.

The 3 compounds were dialyzed against 0.1 *N* NaOH solution (pH 4.5) at 0°C. for periods of 36–48 hr., using Visking cellophane sausage casing.

Each compound was dissolved in 0.1 *N* NaOH and the pH of the solution was adjusted to 4.5 by the addition of sodium acetate, and to 10–11 by KOH.

The fluorophotometric determinations of the pterines were carried out according to the method previously described (4) for riboflavin. The model B Pfaltz and Bauer

fluorophotometer was employed. An 85-watt mercury capillary arc at 110 volts A. C., was used as the exciting light source. A pure blue activating light was produced by the use of filter combinations with a peak between 4300 Å and 4400 Å. The fluorometric photocell was protected by an orange filter, which cut out all wave lengths shorter than 5200 Å. Riboflavin was used as a standard for making quantitative fluorescent measurements of the pterine pigments. The stock riboflavin solution (20 γ /cc. was made up by dissolving 20.0 mg. of riboflavin (Merck's) in doubly distilled water with the addition of a few drops of glacial acetic acid and water up to 1000 cc. Each time fluorescence determinations were made on the pterines, a standard riboflavin solution (0.1 γ /cc.) was made by diluting 1 cc. of the stock solution to 200 cc. The fluorophotometer was checked at the beginning of each set of determinations with the freshly prepared standard solution (0.1 γ /cc.). The readings on the scale of the galvanometer were adjusted so that, with the standard riboflavin solution in the optical cell, a scale value of 35 was obtained. The calculations were carried out as follows: The optical cell containing the unknown pterine was placed in the fluorophotometer, and a reading (F) was made. The standard solution of riboflavin was next placed in the optical cell and a reading (R) made on it. After each reading of the fluorophotometer, a few crystals of sodium hydrosulfite were added to the contents of the optical cell to reduce the riboflavin or pterine, and another reading (B) was made. Then $(F-B)/(R-B)$. rz = pterine/cc. in unknown (r = concentration of pterine in γ /cc. of standard solution; z = dilution factor). Determinations of pterines were carried out at pH 4.5 and 10-11.

In order to make a quantitative spectrophotometric study of the pigments, a sample of each pterine was placed in an ampoule (with cork removed) and the latter was inserted into the chamber of an Abderhalden drying pistol and the apparatus evacuated to a pressure of 1 mm. Hg for a period of 14 hr. at room temperature. After that time the ampoule was transferred to a desiccator and then weighed on the analytical balance. The empty ampoule was returned to the drying pistol and evacuated for 14 hr. as before. The weight of the sample is obtained by the difference in weight of the empty ampoule and of the ampoule plus the pigment. The spectrographic analyses were made with the Beckmann DU spectrophotometer using a hydrogen discharge tube as the source of light for the ultraviolet absorption. All spectrophotometric determinations were made at pH 4.5.

In addition to the normal eggs, X-rayed eggs were used. Four-day prediapause eggs were X-rayed with 1000 Roentgen units in lots of 500 and kept at 25°C. for 4 weeks. At the end of that period, the eggs were placed at 5°C. for another 4 weeks to break the diapause block. By the use of X-irradiation, it has been possible to destroy the embryo and determine the extent to which embryonic *vs.* nonembryonic cells are responsible for the production of these pterines. In general, it was found that these pterines are produced by the embryonic cells.

RESULTS

Analyses of pterines have been made throughout the entire developmental period of the egg. These pterines may be crystallized, and a specific form of crystal is obtained for each compound. The occurrence

of these compounds is not detected at any time during the prediapause and diapause periods. The two light compounds appear during the fourteenth day, and the purplish-brown on the sixteenth day of the postdiapause period.

Absorption analyses of the 3 compounds were made throughout the ultraviolet range.

A comparison of the ultraviolet absorption curve of the purplish-brown compound with the curve obtained by O'Dell *et al.* (10) for dihydrovitamin B₆, indicates some striking similarities in the properties of the two substances. As shown in Fig. 1, the ultraviolet absorp-

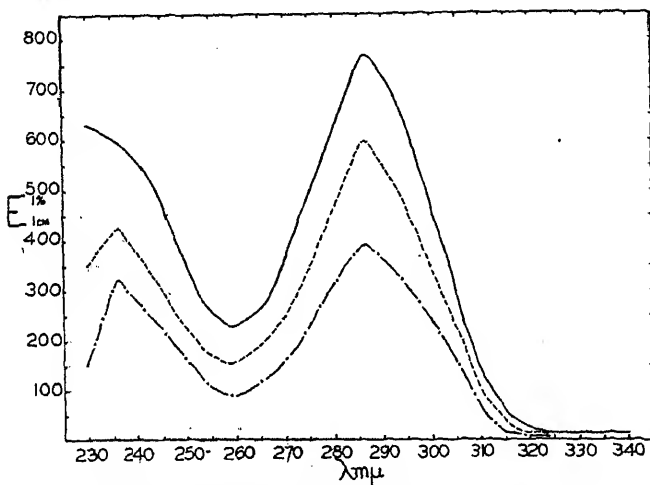


FIG. 1. ——— Ultraviolet absorption curve of the purplish-brown compound at pH 4.5 level; ---- ultraviolet absorption curve of the light-brown compound (aqueous extraction) at pH 4.5 level; - · - · - ultraviolet absorption curve of the light brown compound (alcoholic extraction) at pH 4.5 level.

tion curve of the purplish-brown compound at pH 4.5 has a maximum at 286 mμ. Dihydrovitamin B₆ at pH 11, has a maximum at 284 mμ.

A comparison of the absorption curves for the 2 light brown compounds of the alcoholic and aqueous extractions at pH 4.5 levels brings out some striking similarities. As shown in Fig. 1, the ultraviolet absorption curves for both compounds at pH 4.5 have maxima at 236 and 286 mμ. The shape of the crystals seems to indicate that these compounds are different. In general, the ultraviolet absorption curves for the 2 light brown compounds of Fig. 1 are similar in some respects

to the curve for xanthopterine at pH 7.1 previously described (11). The ultraviolet absorption characteristics of these pterines are somewhat similar, but the relationship of dihydrovitamin B_6 and xanthopterine to these classes of compounds must await further chemical study. The general properties of these isolated compounds are indicated in Table I.

TABLE I
General Properties ^a

Compound	Shape of crystals	E_m	Melting point	Refractive index			2V	Birefringence	Extinction	Principal refractive indices	Interference figure
				α	β	γ					
Light brown (alcoholic extractions) ^b	Diamond shaped	236, 286	Begins decomposing 200°C.								
Light brown aqueous (extraction)	Disc-shaped pellets with furrows	236, 286	Begins decomposing 208°C.	1.50	1.54	1.56	43°56'	0.02	Parallel	Gamma and Beta	+ Biaxial
Purplish-brown	Disc-pellets shaped with furrows	286	Begins decomposing 180°C.	1.62	1.65	1.66	13°54'	0.03	Symmetrical	Gamma and Beta	+ Biaxial

^a The three compounds have the following properties in common: 1. Soluble in 0.1 N NaOH, 0.1 N NaHCO₃, hot 95% alcohol, hot 0.1 N HCl and hot water. 2. Insoluble in cold water, cold 0.1 N HCl, ethyl alcohol, ether and acetone. 3. Diffusible and fluoresce in both acid (pH 4.5) and alkaline (pH 11) media. In acid medium (pH 4.5) they are reducible with sodium hydrosulfite. They are not reducible at pH 11. 4. The murexide reaction is negative.

^b Size of the fragment prevented optical measurements.

Total Fluorescence of the Three Compounds at pH 4.5 and 10-11

An inspection of the data shown in Table II, indicates quite clearly that, as development proceeds, the amounts of alkaline fluorescence (due to the pterines) increases during the late postdiapause to a maximum in the newly hatched nymphs. The total alkaline fluorescence for the 3 compounds at the time of hatching (Table II), approaches that for the original riboflavin found by Bodine and Fitzgerald (4).

Dialysis

The isolated compounds are diffusible and, as such, are recovered quantitatively in the inner and outer solutions of the dialyzing system (Table III).

TABLE II^a

Fluorescence of the Compounds Isolated from the Eggs of 15-Day Postdiapause Period of Development and from the Newly Hatched Nymphs^b

Compound	Total fluorescence (pH 4.5)		Reducible (pH 4.5)		Nonreducible (pH 4.5)		Total fluorescence and nonreducible (pH 10-11)	
	15-Day postdia- pause	Nymph	15-Day postdia- pause	Nymph	15-Day postdia- pause	Nymph	15-Day postdia- pause	Nymph
Light brown (alcoholic ex- traction)	Av. 1.13 S.E. \pm .086	2.84 \pm .115	0.633 \pm .0516	1.54 \pm .058	0.47 \pm .017	1.29 \pm .106	1.32 \pm .103	3.23 \pm .203
Purplish- brown	0.00 0.00	6.91 \pm .358	0.00 0.00	5.21 \pm .321	0.00 0.00	1.64 \pm .131	0.00 0.00	7.13 \pm .931
Light brown (aqueous ex- traction)	6.66 \pm .225	10.90 \pm .549	4.41 \pm .283	9.09 \pm .589	2.24 \pm .110	2.06 \pm .129	7.45 \pm .134	12.07 \pm .517
Complete sum- mation of the three isolated compounds	7.80 \pm .263	20.66 \pm .849	5.04 \pm .305	16.213 \pm .930	2.70 \pm .108	4.99 \pm .149	8.84 \pm .175	23.10 \pm .687

^a All values expressed in galvanometer scale readings; 35.5 = 00.10 γ riboflavin or equivalent per egg or nymph.

^b Each of the values here given represents an average taken from the results of 16 experiments.

TABLE III^a

Results of Typical Dialyzing Experiment of the Three Isolated Compounds^b

Compound	Before dialysis	After dialysis		Total fluorescence	
	Original	Inside of shell	Outside of shell	Before dialysis	After dialysis
Light brown (alcoholic extraction)	0.0835	0.0381	0.0417	2.80	2.60
Purplish-brown	0.2566	0.0186	0.1985	8.60	8.91
Light brown (aqueous extraction)	0.4029	0.0999	0.3163	13.50	13.80

^a Figures expressed in γ of riboflavin per egg, except where total amounts are indicated.

^b Each of the values here given represents an average taken from the results of 5 experiments.

DISCUSSION

The number of pterines that have been described in recent years is surprisingly large. In many cases, their apparent differences are small, and it is not unreasonable to hope that, with further work, these pterines isolated from altogether different sources may prove to be identical. Among earlier workers are Wieland and Schöpf (2), and Schöpf and Becker (12). These authors called attention to the presence of xanthopterine and leucopterine in the wings of *Pieris* (butterfly) and xanthopterine in the integument of a wasp. Erythropterine is present in the red parts of the wings of butterflies of the class *Pieridae* (12). Koschara and Haug (13) observed the presence of xanthopterine in a number of organs and tissues of man and other animals. They reported that it is especially abundant in the liver and kidney. According to these authors there is a certain parallel relationship between the contents of xanthopterine and riboflavin in the organs studied, which seems to indicate that the two substances are functionally dependent.

Numerous observers have reported that the pterine fluorescyanin has a remarkable positive effect on growth of riboflavin-deficient rats (14, 15). These authors (14, 15) used impure extracts of fluorescyanin in which the riboflavin had been removed by irradiation. The results were entirely confirmed when the riboflavin-deficient rats were fed on pure fluorescyanin (16).

Working with *M. differentialis*, Bodine and Fitzgerald (17) pointed out that the riboflavin content of the egg is fairly constant and remains so until late in the egg's development. These workers reported that, late in the development of the egg, some new fluorescent compounds are formed, which are diffusible and fluoresce in both acid (pH 4.5) and alkaline (pH 10) media. These authors suggested that, during the late postdiapause, riboflavin possibly becomes incorporated into other fluorescent compounds.

In the present studies, evidence was obtained along 3 lines, which seems to indicate that these isolated compounds are, in some way, related to the alkaline fluorescent materials previously described (4). (a) These isolated compounds appear during the late postdiapause and show a marked increase during the same period in which the alkaline fluorescent materials of Bodine and Fitzgerald (4) are increasing. (b) The alkaline fluorescent materials and the isolated compounds fluoresce in both acid (pH 4.5) and alkaline (pH 10) media. The isolated

compounds are reducible in an acid medium (pH 4.5). (c) The alkaline fluorescent materials and these isolated compounds are diffusible and are recovered quantitatively in the dialyzing system.

It seems possible that the riboflavin molecule, in some way, may serve as a nucleus for the newly formed fluorescent compounds. The present interpretation is consistent with that of Bodine and Fitzgerald (4).

The results of the present studies of the isolated compounds produced during the course of the development in the grasshopper egg, lead one to consider them to be pterines. Their physicochemical properties, such as fluorescence and ultraviolet absorption, are quite similar to those described for pterines (3). The exact chemical mechanisms of the origins of pterines are at present poorly understood.

As to the origin of these isolated pterines, several explanations seem possible. First, synthesis of the pterines and use of the riboflavin could be in competition with each other for the same substrate during the earlier development of the egg. In the late postdiapause a condition may be established in the developing egg which would permit the synthesis of pterines and the retardation of the use of riboflavin. Second, a transformation of riboflavin, in some way, to pterines seems possible. The latter of these suggestions seems more probable in the light of the findings of Bodine and Fitzgerald (4, 17). These authors (4, 17) demonstrated that the original fluorescent material, riboflavin, is inherent in the egg and remains constant in quantity until the late postdiapause. As the embryo grows in size, increasing amounts of riboflavin or its derivatives leave the yolk and enter the embryo (4). A transformation, in some way, of riboflavin to pterines could possibly occur as the former leaves the yolk to enter the embryo proper. The true identity and the actual mechanisms of their formation must await further study of the chemical constitution of these compounds. Attention is being given to this point and the results will be reported at a later date.

The physiological functions of pterines are not well understood. Some of them possess a high oxidation—reduction potential which makes it possible for them to play, in some way, an essential role in cellular oxidation. The investigation of the compounds herein reported is at a stage where few final conclusions can be drawn concerning their chemical identity, possible origins and physiological functions. Such correlations and explanations must await further study.

SUMMARY

1. A preliminary quantitative study has been made of three pterine compounds in the egg of the grasshopper (*M. differentialis*) throughout its entire development.

2. One purplish-brown and two light brown compounds were isolated from the egg during the late postdiapause period of the egg's development.

3. The specific ultraviolet absorption curves are presented.

4. The compounds are diffusible and fluoresce in both acid (pH 4.5) and alkaline (pH 10) media. In an acid medium (pH 4.5) they are reducible.

5. The compounds occur during the late postdiapause and show a marked increase that continues during the subsequent development of the egg.

6. The physicochemical properties, such as fluorescence and ultraviolet absorption, are quite similar to those described for pterines.

7. As pointed out, it seems that the riboflavin molecule may serve as a nucleus for pterines during the late development of the egg.

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The Biosynthesis of Auxin in the Vegetative Pineapple.

I. Nature of the Active Auxin

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INTRODUCTION

Evidence is accumulating that one of the significant factors controlling floral initiation in the pineapple is free auxin level (5, 9). This made a study of the nature and origin of the native auxin pertinent to the work in this laboratory on pineapple flowering. Moreover, the subject of auxin nature and origin is of wide physiological interest. The present paper deals with the identity of the active auxin in the pineapple. It will be shown that two forms of auxin are present, an acid form which possesses the characteristics of indoleacetic acid (IAA), and the neutral form which is probably indoleacetaldehyde.

GENERAL METHODS

The bases of the youngest leaves of the Cabezona pineapple were used in the present work unless otherwise noted. These tissues are relatively low in free auxin and high in their contents of auxin precursor³ (8). Chilled leaf bases were rapidly cut into small pieces and dropped into diethyl ether. The free auxin was obtained by 2 or 3 0.5 hr. extractions in the dark at room temperature, while the auxin released in the subsequent 20 hr. of extraction was considered as arising from its precursor (8). About 30 g. of leaf material were taken from each plant. All of the ether used was freshly purified by shaking with and distilling from an aqueous mixture of $\text{FeSO}_4\text{-Ca(OH)}_2$.

For separation of the acid and neutral components of an extract, the ether was reduced in volume to about 100 ml. and partitioned 4 times with 20 ml. of saturated glucose solution plus 2 ml. of 8% NaHCO_3 . Glucose was employed to facilitate separation of the ether and aqueous phases after partitioning. The combined aqueous layers were washed twice with fresh ether and these washes were added to the original ether.

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³ The substance or substances which are readily converted to free auxin at physiological temperatures and pH levels.

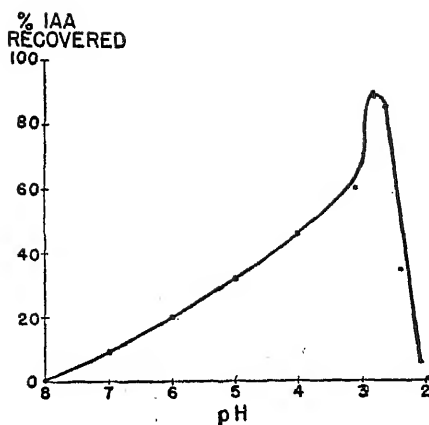


FIG. 1. Partition characteristics of indoleacetic acid. 0.1 γ IAA in 25 ml. water partitioned 3 times with 3 vols. ether after pH adjusted to indicated values with HCl or NaOH.

Practically all of the neutral fraction remained in the ether, while the acid fraction was obtained by acidification of the combined aqueous phases to pH 2.8⁴ with HCl and then immediately partitioning 3 times with 2-3 volumes of ether.

An ether extract to be assayed was reduced in volume by distillation, drop-pipetted into a small vial, and evaporated to dryness. To the residue 1.5% agar was added, generally 1 ml., and the auxin allowed to diffuse overnight before dilution and assay.

The standard *Avena* test as modified for tropical conditions (7) was used for auxin determinations. For each experiment a sensitivity curve was obtained by assay of known concentrations of crystalline IAA. Test curvatures were then converted to equivalent concentrations of IAA by reference to this curve and expressed as $\gamma \times 10^{-3}$ IAA. All of the following auxin values are based on curvatures within the linear dilution range, the appropriate range being determined by dilution of the original volume of agar.

RESULTS

Acid Auxin

To ascertain the identity of the acid auxin in the pineapple, two methods of approach were possible: either direct isolation, or com-

⁴ The pH of 2.8 was chosen since it was found that at this pH maximum recovery of IAA could be secured (cf. Fig. 1). This value agrees with what could be theoretically expected from the dissociation curve of IAA at different pH levels (13). The sharp drop in IAA recoveries at the more acid pH levels may be due to concomitant acid inactivation of the auxin. A similar low recovery at low pH values was observed when tartaric acid was employed instead of HCl for acidification.

paring the properties of the native auxin with the properties of auxins already identified in higher plants. Since auxin levels are relatively low in pineapple leaf tissues, the second procedure was used, which comprised determinations of the pH sensitivity, enzymatic inactivation, rate of diffusion, and activity curve of the native auxin.

pH Sensitivity. The 3 acid auxins of higher plants which have been characterized, auxin *a*, auxin *b*, and indoleacetic acid, have different sensitivities to hot acid and alkali. IAA is relatively stable in alkali but is sensitive to acid, auxin *a* is inactivated by alkali but is stable in acid, while auxin *b* is unstable at both pH extremes (3). Accordingly, both the free auxin of the leaf and crystalline IAA were refluxed with hot acid and alkali using, with some modifications, the procedure of van Overbeek and Bonner (6).

A free auxin ether extract was divided into 4 equal parts. The ether was reduced in volume, transferred to micro Kjeldahl flasks, and evaporated over warm water. To two of the flasks 10 ml. of water were added, to the third and fourth, respectively, 10 ml. *N* HCl and *N* NaOH. Excluding one with water as an untreated control, the remaining solutions were refluxed 2 hr. in boiling water. The pH was then adjusted to 2.8 and the auxin removed by partitioning with ether. Exactly the same procedure was followed with 0.2 γ crystalline IAA being added to the flasks instead of leaf auxin.

Table I shows that the two auxins have like pH sensitivities. Complete inactivation resulted from the acid treatment, while alkaline reflux destroyed roughly $\frac{3}{4}$ of the activity of both the native and synthetic auxins. Although IAA has been considered to be relatively stable in hot alkaline solution, the partial inactivation resulting from prolonged heating in alkali agrees with some recent observations (4, 2), particularly when the auxin is unprotected by proteins (1).

TABLE I
pH Sensitivities of the Pineapple Free Auxin and Indoleacetic Acid

	Refluxed in:			
	—	H ₂ O	HCl	NaOH
Pineapple auxin $\gamma \times 10^{-3}$	78	56	0	22
Per cent recovered	100	72	0	28
Indoleacetic acid $\gamma \times 10^{-3}$	99	48	0	20
Per cent recovered	100	49	0	20

Enzymatic Inactivation. Tang and Bonner (11) have described an enzyme system obtained from etiolated pea epicotyls which inactivated IAA by oxidation of the side chain. The enzyme appears to be highly specific, not attacking a variety of other auxins or auxin analogs, although the effect on auxins *a* and *b* has not yet been determined. The sensitivity of the native auxin to this enzyme was therefore investigated.

About 30 g. etiolated pea seedlings, 12-15 cm. in length, were ground to a smooth paste with quartz sand and water. The dispersion was centrifuged and the enzyme precipitated by the slow addition of acetone in a ratio of 4 parts acetone to 10 parts supernatant.⁵ The precipitate was recovered by centrifugation and redispersed in 25 ml. 0.1 *M* KH_2PO_4 - Na_2HPO_4 buffer, pH 6.8. As far as practicable all solutions were maintained at 5°C.

To determine the effect of the enzyme on the native auxin, a free auxin ether extract of leaf tissues was reduced in volume, transferred to a 250 ml. Erlenmeyer flask, and evaporated to dryness by warming slightly. To the residue 94 ml. 0.1 *M* KH_2 - Na_2HPO_4 buffer, pH 6.8, were added and then 6 ml. of the enzyme dispersion. The mixture was incubated for 16 hr. in the dark (27°C.) with constant agitation. Several drops of CHCl_3 were added before the incubation as a preservative. For the recovery of the auxin, the solution was adjusted to pH 2.8 and then partitioned with ether. As a check on the enzyme activity, 0.2 γ IAA were treated in an identical manner. As controls, both the leaf auxin and IAA were incubated in buffer without enzyme.

TABLE II
*Inactivation of Pineapple Free Auxin and Indoleacetic Acid
by the Indoleacetic Acid-Inactivating Enzyme of Pea*

	$\gamma \times 10^{-3}$ recovered		
	Control	+ Enzyme	+ Boiled enzyme
Leaf auxin	400	0	370
IAA	140	0	120

Table II, the data of a typical experiment, shows that both the free auxin of the pineapple leaf and IAA are completely inactivated by the enzyme preparation. When the enzyme was heated to 100°C. for 4 min. previous to incubation, no significant inactivation of the auxins occurred.

Rate of diffusion. The diffusion test as a means of determining the molecular weight of an auxin has been frequently described in detail

⁵ J. Bonner, personal communication.

((2) and (4), for example). Basically, the method consists of allowing the auxin in a donor block of agar to diffuse through a bank of plain agar blocks, and then determining the distribution of the auxin after a given time. Thus, similar relative distributions are to be expected from substances of like molecular weights. Diffusion experiments were therefore performed comparing the acid fraction of free auxin extracts of leaf bases and crystalline IAA.

Approximately 0.15 γ of the free acid auxin of the leaf or 0.1 γ IAA was taken up in 1 ml. of 1.5% agar. From each agar two donor blocks ($14.0 \times 14.2 \times 1.42$ mm.) were poured, and each donor block was placed upon a bank of 3 plain agar blocks of like dimensions. Diffusion was allowed to proceed for 2 hr. in a moist atmosphere at 28°C. The blocks were then separated, cut into 16 equal parts, and assayed. The *Avena* curvatures obtained were converted to γ -equivalents IAA using the IAA activity curve. From these values the percentage distribution of actual concentrations of auxin in each block was calculated.

TABLE III
Average Values of Auxin Distribution in Diffusion Experiments
A-Leaf auxin B-Indoleacetic acid

Block	γ /l. (from activity curve)		Per cent distribution	
	A	B	A	B
I	40.0	26.0	37.5	35.9
II	30.0	21.0	28.2	29.0
III	22.5	15.5	21.1	21.4
IV	14.0	10.0	13.2	13.8

The average data of replicated values are given in Table III, where it is apparent that the relative distributions of the two auxins are extremely close. Hence, we can conclude that the rates of diffusion and, therefore, the molecular weights of both auxins are very similar.

From the graphic representation of Kawalki's tables in (4), which relate the diffusion coefficient of a substance to its distribution in 4 layers, diffusion coefficients (k) of the two auxins can be calculated. For the distribution data of Table III, $k = 0.536$ for IAA, while $k = 0.527$ for the auxin of the leaf. Utilizing the formula of Öholm, wherein the diffusion coefficient of a substance is related to its molecular weight,

$$k_1\sqrt{M_1} = C = k_2\sqrt{M_2},$$

M for IAA being 175, a value of 181 is obtained for the molecular weight of the leaf auxin.

Activity Curve of the Acid Auxin. An activity-concentration curve of the leaf auxin was constructed from assays of replicate aliquots taken from the acid fraction of a free auxin extract. As can be seen in Fig. 2, the curves of the leaf auxin and synthetic IAA are similar. In addition, *Avena* curvatures of the acid auxin of the leaf are like those produced by IAA, *i.e.*, they embrace the upper halves of the coleoptiles and are not restricted to the tips. This characteristic is unlike that of numerous other auxin analogs and homologs, which likewise have smaller orders of activity than IAA (4). Although this type of delimitation does not exclude auxins *a* or *b*, the previously detailed experiments make untenable the probability that an appreciable portion of the native acid auxin is either auxin *a* or *b*.

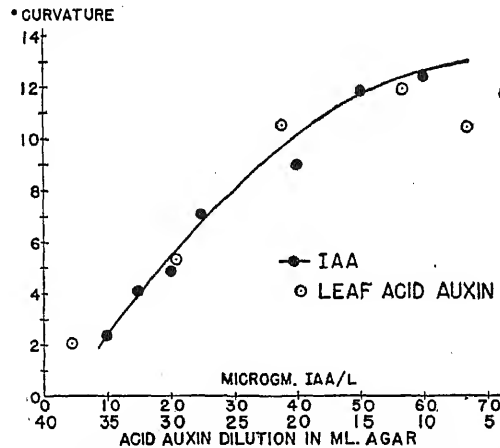


FIG. 2. Activity-concentration curves of the acid fraction of the leaf free auxin and of indoleacetic acid.

Neutral Auxin

Larsen (4) has shown that a number of plants, particularly etiolated peas and cabbage, contain a neutral substance which can be oxidized to IAA by soil, raw milk, and by preparations of Schardinger enzyme. Hemberg (2) has found that this substance also occurs in the potato tuber. Based on its molecular weight as determined by diffusion, its conversion to IAA by aldehyde systems, and the fact that a product of similar properties could be obtained from tryptophan treated with

isatin or ninhydrin, Larsen concluded that the neutral substance was indoleacetaldehyde (IAc). He called it a neutral auxin because of the activity manifest by unconverted neutral fractions in the *Avena* test. The likelihood that this neutral form occurs in the pineapple leaf was then investigated.

Conversion of the Neutral Fraction to Active Auxin. A 24 hr. ether extract of leaf tissues was separated into its neutral and acid fractions. The ether phase (containing the neutral fraction) was divided into two equal parts, transferred to small Erlenmeyer flasks, and evaporated to dryness over warm water. One part was incubated with soil according to the procedure of Larsen. The other part was treated in an identical fashion, except that the soil was omitted. Other neutral fractions were similarly obtained and treated with raw milk or Schardinger enzyme following Larsen's techniques. After incubation, the media were acidified to pH 2.8 and partitioned with ether, the ether then being assayed for auxin content.

TABLE IV

Conversion of the Neutral Fraction of Pineapple Leaf Extracts into Acid Auxin
No activity was obtained from the conversion agents incubated alone

	γ IAA $\times 10^{-3}$			
	Soil	Milk	Schardinger enzyme	
Extract alone	1.5	1.8	2.0	3.0
Extract+conversion agent	180	210	190	170

The results given in Table IV show that the pineapple leaf possesses the neutral component which is converted to acid auxin by soil, milk, or Schardinger enzyme. It was repeatedly observed that free auxin extracts of the leaf did not contain detectable amounts of the neutral substance. Only when the extraction was prolonged could appreciable quantities of the neutral substance be secured.

pH Stability. According to Larsen (4) the neutral substance is heat stable in neutral solutions while it is completely inactivated by boiling with either acid or alkali. To check this characteristic for the neutral form in the pineapple leaf, we subjected neutral fractions to water, acid, and alkali reflux for 1 hr. in a manner similar to that described for the acid auxin. The refluxed extracts were then assayed for neutral hormone content by conversion to the acid form with soil. There was but slight inactivation by neutral reflux and marked or complete destruction in both the acid and alkali treatments, similar to Larsen's

results. However, small but definite negative curvatures restricted to the tips of the *Avena* coleoptiles resulted in the assay of the acid- and alkali-treated samples. These were unlike the curvatures normally obtained and make us hesitant to assign definite concentration values for these experiments.

In regard to the lability of the neutral substance, we have found, as did Hemberg (2), significant destruction to occur on shaking ether extracts with acidified glucose solutions. Moreover, no neutral substance was obtained by either a short or prolonged ether extraction of fresh leaf material which had been dried at 80°C. under forced draft. The latter results are probably also due to an inactivation of the system responsible for the origin of the neutral form, as well as a direct inactivation.

Aldehyde Nature of the Neutral Form. Reaction with Dimedon. While Larsen's evidence that the neutral substance is indoleacetaldehyde is logical, we felt that further substantiation of its aldehyde nature was desirable. For the experiments detailed below, neutral fractions from the young roots of both pineapple and *Taraxacum officinale* (Weber) were used. These organs have relatively high concentrations of the neutral substance, *Taraxacum* roots in particular being a rich source.

Dimedon (5,5-dimethylcyclohexanedione-1,3) is a sensitive aldehyde reagent which does not react with ketones (12). The reactivity of the neutral substance to dimedon was therefore tested.

A 24 hr. ether extract of 120 g. young roots was separated into its acid and neutral fractions. The neutral fraction in ether was divided in half, transferred to 25 ml. Erlenmeyer flasks, and evaporated to dryness over warm water. The residues were then taken up in 5 ml. 0.1 *M* acetate buffer, pH 5.0, by shaking 5 min. To one flask 0.5 ml. of a saturated aqueous dimedon solution (≈ 2 mg. dimedon) was added, the other flask serving as a control. After 10 min. agitation, the solutions were placed in the dark at room temperature for 20 hr. They were then partitioned with ether and the ether extracts incubated with soil in the usual manner for assay of the neutral substance.

From a neutral fraction of pineapple roots treated with dimedon 4×10^{-3} γ IAA were recovered, while its comparable untreated control yielded 67×10^{-3} γ . Likewise, after dimedon treatment of a *Taraxacum* neutral fraction 22×10^{-3} γ IAA were obtained, whereas its control assayed 185×10^{-3} γ . Reductions in level of the neutral form of, respectively, 94% and 88% on exposure to dimedon may be interpreted as a substantiation of the aldehyde nature of the neutral substance.

The above experiment was repeated with crystalline IAA instead of the neutral form. No significant difference in activity resulted from the incubations with and without dimedon, indicating that IAA is not affected by the aldehyde reagent under

these conditions. In addition, the activity of IAA in the *Avena* test was unaffected by the presence of dimedon in the agar block.

Reaction with Bisulfite. A well known organic reaction is the addition of sodium bisulfite to aldehydes in aqueous solution. The addition product is insoluble in ether, and can be regenerated to the original aldehyde by sodium carbonate. The reaction takes place with all aldehydes. Besides aldehydes, only aliphatic methyl ketones or ketones containing two methylene groups adjacent to the carbonyl are able to form the bisulfite addition product, and these but slowly (10).

The neutral fraction from a 48 hr. ether extraction of 87 g. *Taraxacum* roots was treated with sodium bisulfite following the procedure outlined in Fig. 3. The data given in the figure show that the neutral

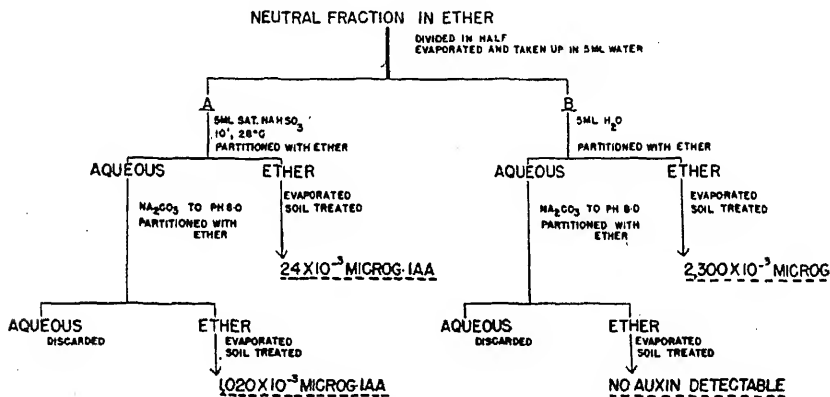


FIG. 3. Formation and regeneration of the bisulfite addition product of the neutral substance.

substance will form the bisulfite addition product, and that the original form, which is convertible by soil to IAA, can be regenerated by the addition of sodium carbonate. A 45% recovery of the original activity was obtained on regeneration. Recoveries up to 90% were later secured with neutral fractions of pineapple leaf bases and roots by increasing the bisulfite reaction time to 5 hr. We consider this to be supporting evidence that the neutral substance is a carbonyl compound.

DISCUSSION

The present experiments show that two forms of auxin exist in the leaf tissues of the pineapple plant—an acid form and a neutral form.

The former is directly active in the *Avena* test and can, therefore, be correctly termed the acid auxin. Its characteristics are very similar to those of IAA. It has the same partition behavior, a like sensitivity to acid and greater stability in alkali, is inactivated by the IAA inactivating enzyme of pea, has virtually the same rate of diffusion in agar, and possesses the same order of activity and type of *Avena* curvatures. Thus, it is highly probable that the active acid auxin is indoleacetic acid.

IAA has been indirectly characterized in other higher plants by numerous investigators and has been actually isolated from corn, although there is still some reluctance to classify it categorically as a native plant hormone (1). It is pertinent that no indications of either auxins *a* or *b* were obtained in the present work.

The neutral form identified by Larsen as indoleacetaldehyde also occurs in the pineapple. We have favored the term neutral substance or form in this paper, rather than neutral auxin, since the evidence is not conclusive that this form is directly active in the *Avena* test. We could obtain little or no activity when neutral fractions were assayed directly, whereas high auxin activity arose on conversion by soil, milk, or Schardinger enzyme to IAA. Moreover, some of Larsen's neutral fractions likewise yielded no activity upon direct assay, but were highly active after conversion. It is possible that the activity which is sometimes manifest by clean preparations of a neutral fraction arises from a rapid enzymatic conversion in the *Avena* coleoptile.

The inactivation of the neutral form by dimedon, and its ability to form a bisulfite addition product from which it can be regenerated, substantiate its aldehyde nature. Although the bisulfite addition does occur with some ketones, the fact that IAA is obtained on conversion of the neutral form by aldehyde systems tends against the ketone possibility. These reactions, supplementing Larsen's evidence, strengthen the likelihood that the neutral form is indoleacetaldehyde, though direct isolation and characterization are necessary for conclusive proof.

As was mentioned before, the neutral substance could only be obtained upon prolonged extraction of the cut leaf tissues. It could not be detected in the free auxin ether extract. Apparently the neutral form does not exist in appreciable concentrations in a readily extractable state, as does the acid auxin. It may be more slowly formed or liberated in the tissues, and/or be rapidly transformed as it arises. Under the last possibility, the continuous presence of ether would enable a con-

current diffusion of the neutral form out of the tissue and away from enzymatic attack.

SUMMARY

1. Two forms of auxin were shown to occur in the pineapple leaf: a neutral and an acid form.

2. The pH sensitivity, inactivation by the indoleacetic acid inactivating enzyme of pea, rate of diffusion, and order of activity of the acid auxin indicate that it is indoleacetic acid.

3. The neutral form is similar to the neutral substance described and characterized by Larsen as indoleacetaldehyde.

4. The aldehyde nature of the neutral form was substantiated by its inactivation with dimedon and the formation of a bisulfite addition product from which it could be regenerated.

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The Biosynthesis of Auxin in the Vegetative Pineapple.

II. The Precursors of Indoleacetic Acid

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INTRODUCTION

When cut pineapple tissues are immersed in ether, auxin is rapidly extracted. After 1-2 hr. of extraction comes a lag period, the auxin yield being negligible, and then the rate of yield again rises. This phenomenon has been used as a basis for separating the free active auxin initially present from the free auxin which subsequently arises from a reserve form or precursor (16)³.

As shown in the preceding paper (8), there is strong evidence that the active acid auxin in the vegetative pineapple is indoleacetic acid (IAA), and that the neutral substance indoleacetaldehyde (IAc), which is readily converted to IAA, is also present.

The first ether extraction of pineapple tissues to secure the free auxin contains no detectable IAc, while the ether extract for precursor auxin possesses considerable quantities of the neutral form. This suggested the possibility that IAc was related to the process of auxin biosynthesis, either IAc being the immediate precursor of IAA, or both compounds being formed independently from a common precursor. The former alternative was likewise considered by Larsen (12) in discussing a possible mechanism for the formation of IAc in plants. On the other hand, it has been frequently noted that increased auxin or IAA levels occur in plant tissues in the presence of tryptophan, and therefore this amino acid has been considered a precursor of IAA (2,13,15,22,24,28).

In the present paper it will be shown that IAc can and probably does function as the immediate precursor of IAA in the leaves of the pine-

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³ For convenience in discussion, the free auxin which appears in a tissue after the free auxin initially present has been removed will be termed "precursor auxin."

apple plant, and that this reaction can be experimentally integrated into the concept of IAA arising from tryptophan.

GENERAL METHODS

Unless otherwise noted, only the basal 5 cm. of the youngest leaves of the Cabezona pineapple were used throughout this work. This region is highly etiolated, and includes the meristematic area of each leaf. Plants were taken directly from the field and placed under refrigeration 1–2 hr. before use. All incubations were run in the dark at room temperature (27–28°C.).

Leaf Brei. Leaf bases were rapidly cut into small pieces and blended 5 min. with ice cold water or 0.005 *M* NaCN in a prechilled Waring blender. The mixture was again chilled and redispersed for 5 min. For each 25–30 g. of leaf tissue, 50 ml. of water or cyanide solution were used.

After experimental treatment, the auxin in a brei was extracted by mechanical agitation with 2 vol. ether for 30 min., the ether being replaced every 5 min. with fresh solvent. Where required, separation of the acid and neutral components of the ether extract was carried out by partitioning with glucose and bicarbonate solutions as previously described (8).

Leaf Disc Infiltration. Generally 200 discs, 1 cm. in diameter, were cut from the bases of the leaves. Each disc was cut in half, and the halves placed in two 25 ml. filtering flasks. Fifteen ml. of the desired solution were added and infiltrated into the discs by four 5 min. evacuations by water aspirator. After incubation for 4 hr., the discs were washed with distilled water and transferred into ether. Acid and neutral fractions of the ether extract were separated as noted above.

Leaf Enzyme Mixture. Approximately 1.5 kg. of young leaf bases were chilled, cut into small pieces, and frozen at –12°C. They were then blended into 5 l. of cold 0.1 *M* KH_2PO_4 – Na_2HPO_4 buffer, pH 7.0. The brei was centrifuged in a basket head jacketed with “sharkskin” paper and the filtrate passed through a Sharples super-centrifuge. To the clear supernatant, solid $(\text{NH}_4)_2\text{SO}_4$ was added (68 g./100 ml. liquid) and dissolved. After standing overnight at 4°C., the flocculated protein was recovered in the Sharples, and then dialyzed in sealed Cellophane tubing against frequent changes of distilled water for 30 hr. at 2°C. Following dialysis the dispersion possessed a solid content of 54 mg./ml.

For incubation with the crude enzyme, the substance to be treated was taken up in 50 ml. 0.1 *M* KH_2 – Na_2HPO_4 buffer of the desired pH. One ml. of the enzyme dispersion was added and the mixture gently shaken for 10 min. All incubations with the enzyme were of 4 hr. duration. Unless otherwise stated, acid and neutral fractions were obtained after incubation as follows: The pH of the medium was first adjusted to 8.0 with 8% NaHCO_3 solution, and the neutral fraction then removed by partitioning 3 times with 3 vol. of ether. The acid fraction was secured in a like manner after the pH of the aqueous phase was brought to 2.8 with HCl.

Preparation of Indoleacetaldehyde. It has been demonstrated (8) that IAc forms a bisulfite addition product from which the aldehyde can be regenerated by Na_2CO_3 . This procedure was followed in the present work in order to employ partially purified preparations of IAc.

As a source of the neutral substance the young roots of both pineapple and *Taraxa-*

cum officinale (Weber) were used. Roots were cut into pieces of less than 1 cm. in length and placed under ether for several days. The neutral fraction of this ether extract was evaporated to dryness in a small flask and the residue taken up in 10 ml. of water by agitation for 3 hr. Ten ml. of saturated NaHSO_3 solution were then added plus solid NaHSO_3 to saturate the entire solution and the mixture shaken 15 min. (It was later found that greatly increased yields could be obtained by extending the reaction time to 3–5 hr.) After partitioning with ether several times to remove lipoidal matter, the neutral form was regenerated and recovered from the bisulfite addition product by bringing the pH to 8.0 with 20% Na_2CO_3 solution and then extracting with ether.

Assay of Indoleacetaldehyde. To estimate the concentration of IAe in a given extract, the neutral form was first converted to IAA by treatment with soil (12). We have used soil as the conversion agent since it was easier to prepare and manipulate, and proved more uniform in activity than either milk or crude Sehardinger enzyme. The neutral fraction of an ether extract to be assayed was evaporated to dryness in a 25 ml. Erlenmeyer flask by gentle warming. Five ml. of water were added and the flask continuously agitated for 1 hr., any residue being dispersed in the water with a glass rod after 10 min. shaking. One g. of local soil (Catalina clay), washed by centrifugation according to (12), and previously suspended in 5 ml. of water, was added to the flask. Shaking was then continued for 1 hr. Thereupon, the mixture was filtered through Whatman No. 41 under suction and the residue washed with water (3×5 ml.). To remove the acid auxin for assay, the filtrate was brought to pH 2.8 with 0.1 *N* HCl and immediately partitioned 3 times with 4–5 vol. of ether.

All of the values for IAe here presented are corrected for the conversion efficiency of the soil used. This approximate factor was determined as follows: Since there was no way of directly assaying the absolute amount of IAe in a neutral fraction, a preparation of IAc was converted to IAA by treatment with soil as just described. The pH of the filtrate was adjusted to 8.0 with 8% NaHCO_3 solution. Unconverted IAe was then removed by partitioning with ether. The remaining aqueous phase was acidified to pH 2.8 and again partitioned with ether to obtain for assay the IAA which arose from the conversion by soil. The unconverted IAc was retreated with soil and the IAA obtained from this second conversion was likewise assayed. The two values of IAA were utilized to derive a conversion ratio by considering them as units of a simple progression:

Let x_1 = conversion ratio of the soil, first incubation,
 a = amount of IAc originally present,
 b = amount of IAc converted in first incubation,
 $a - b$ = amount of IAc not converted in first incubation,
 $(a - b)x_2 = c$ = amount of IAe converted in second incubation.

Assuming $x_1 = x_2$, since the soil catalyst is present in excess amount, and since no increase of IAA results by longer incubation of the IAc than the customarily used 1 hr. period,

$$\text{then } \frac{b}{a} = x_1 = \frac{c}{a - b},$$

$$\text{and } a = \frac{b^2}{b - c},$$

from which x_1 can be calculated.

In a typical experiment, b equaled 1.2γ and c equaled 0.36γ IAA, giving a conversion ratio of 0.7.

Since the method of estimating IAc is based on the yield of IAA after conversion, an additional complicating factor is the partial inactivation by soil of IAA. A series of known amounts of IAA, ranging from 0.03 – 1.0γ , were treated with soil by exactly the same procedure as used for routine conversions. The average loss in activity of 9 determinations was $62 \pm 6\%$. Accordingly, IAc yields obtained were likewise corrected by dividing by 0.38.

Auxin Assay. The method used for the estimation of acid auxin or IAA has been described (8). All auxin concentrations are expressed as γ IAA, and are to be interpreted as γ equivalents IAA as computed from simultaneously determined activity curves of synthetic IAA.

RESULTS

Enzymatic Nature of the Auxin Production. The free and precursor auxin of pineapple leaf brei can be delimited in a similar fashion as for leaf tissues cut into small pieces (16). A continuous ether extraction of brei shows the characteristic drop in the rate of auxin yield after 1–2 hr., followed by a rise in rate. Boiling or heating has been shown to effectively halt this continued formation of auxin in *Lemna* (23) and in tomato leaves and *Iris* ovaries (9). Similar results were obtained with cut sections of pineapple leaves (16). We considered it desirable to substantiate and extend these results with the pineapple leaf. Accordingly, the effect of boiling on the rate of active auxin production from its precursor in breis was investigated. Precursor auxin yields were determined by periodic extractions of breis composed of equivalent amounts of boiled, unboiled, and a combination of boiled and unboiled leaf tissues.

Leaf bases were cut into small pieces and divided into 2 equal parts of 50 g. One part was dropped into boiling water and boiled 10 min. It was then dispersed in the same water by blending as described under general methods. The remaining part was dispersed directly in $0.005 M$ NaCN solution. One-half of the boiled and unboiled breis, each equivalent to 25 g. of the original tissues, were placed directly under ether. The remaining two halves were first mixed before the addition of ether. Free auxin was removed from the 3 preparations by 3 consecutive 0.5 hr. ether extractions under continuous agitation. The breis, in stoppered flasks, were then stored in the dark and periodically extracted with ether. These ether extracts were reduced in volume and viald for auxin assay. Cyanide was used in the unboiled tissue dispersions, since it was found that low or erratic auxin yields were obtained in its absence.

Fig. 1, in which the cumulative yields are plotted, shows that boiling stops auxin production, while in the unboiled brei auxin release continues to some extent. However, from the combined boiled and unboiled

tissue breis greatly increased yields result as compared to the yields from boiled or unboiled breis alone, or to the arithmetic sum of the latter two. The increase is particularly manifest between 1 and 4 days; after 4 days the rates of auxin production drop to negligible values.

These data may be interpreted to indicate that the auxin release in the breis is an enzymatic process, and that, in the unboiled material, the amount of substrate is the limiting factor, rather than the available enzymes involved. The addition of boiled tissue, with the enzymes probably heat-inactivated, can be simply explained as an increase in the amount of utilizable substrate. On this basis, the fact that the yield from the combined boiled and unboiled tissues is roughly twice that of

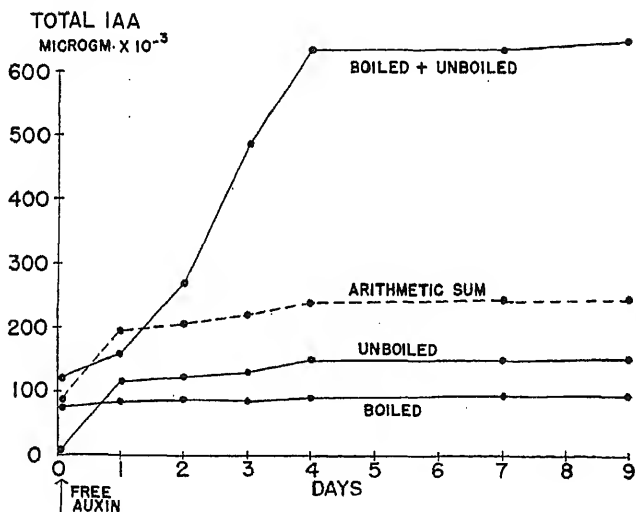


FIG. 1. Auxin yields obtained by periodic extraction of equivalent amounts of boiled, unboiled, and a mixture of boiled and unboiled leaf breis.

the sum of the separate components implies that no significant portion of the substrate was inactivated by the boiling treatment.

Relative Levels of Indoleacetaldehyde and Precursor Auxin in the Whole Plant. To explore further the indication that IAc was related to the formation of auxin, the levels of IAc and precursor auxin in different parts of the pineapple plant were determined.

The various tissues listed in Table I were chilled, cut into small pieces, and dropped immediately into ether. The free auxin extract was discarded, while the IAA level

in the acid fraction of the subsequent 15 hr. extraction was taken as an estimate of precursor auxin. The neutral fraction of this ether extract was treated with soil to ascertain the IAc concentration.

Table I shows that a distinct correspondence exists in the different tissues between the IAc level and the IAA originating from its precursor. It is evident that larger amounts of IAc are found in the extracts of those tissues which are able to produce relatively greater amounts of auxin. The correlation coefficient between the two components is *ca.* 0.87.

TABLE I
Comparative Levels of Indoleacetaldehyde and Indoleacetic Acid Arising from its Precursor in Various Tissues of the Vegetative Pineapple

		γ /kg. fresh weight tissue	
		IAc	IAA
Young leaves	Apex	1.5	0.8
	Base	11.4	11.0
Semi-mature leaves	Apex	0.2	0.1
	Base	1.2	2.2
Mature leaves	Apex	0.4	0.1
	Base	0.3	0.2
Axis	Apex	1.8	1.1
	Center	0.4	0.2
Young roots		15.9	21.8

$$r_{xy} = \frac{\Sigma(x-\bar{x})(y-\bar{y})/N}{\sqrt{V_x \cdot V_y}} = 0.87$$

The relatively high concentrations of IAc in root tissues has not, to our knowledge, been noted before. We found the same to be true for the young roots of *Taraxacum officinale*, several of the chicories, and, to some extent, in the banana (*Musa sapientum* L.).

Comparative Rates of Indoleacetaldehyde and Precursor Auxin Formation in Leaf Breis. The connection between IAc and precursor IAA was also implied by rate of extraction data obtained by periodic assays of the levels of both components in leaf breis.

Twenty-five g. of leaf tissue were blended in cyanide solution. After removal and discard of the free auxin, the brei was placed in the dark under ether and periodically extracted. Each ether extract was separated into its acid and neutral fractions and assayed as usual for IAA and IAc.

When the rates of yield/hr. of both IAc and IAA are plotted (Fig. 2), an inverse relationship between the two rates for the first 96 hr. of extraction is obvious. The progressive decrease in the rate of IAc yield is almost matched by a like increase in the IAA rate. As long as appreciable IAc is present, IAA formation goes on with increasing rapidity. When the IAc drops to negligible levels (96 hr.), IAA levels drop sharply. The inference may, therefore, be drawn that IAA formation proceeds at the expense of IAc.

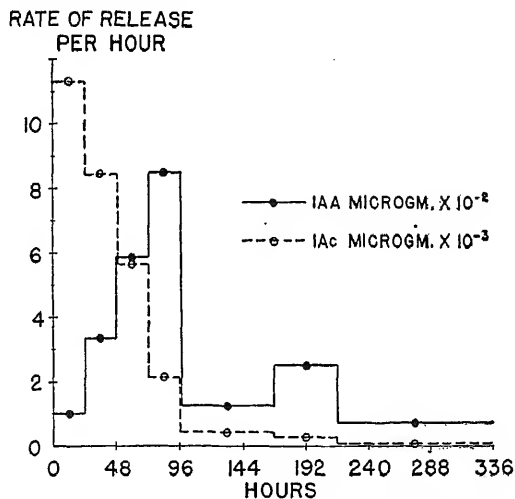


FIG. 2. Rates of release of indoleacetaldehyde and indoleacetic acid from leaf breis.

Since a weak cyanide solution was used in the blending to minimize IAA inactivation (*cf.* below), the possibility of cyanohydrin formation should be considered as an explanation of the diminishing IAc yields. Dispersion in the presence of cyanide should theoretically permit some degree of reaction with carbonyl compounds in the tissues, which would include IAc, as long as free CN^- is present. Since the pH of the leaf brei is about 5.0, a removal of the cyanide by the ether would be expected during the course of the extractions. Both the aqueous and ether phases were, therefore, tested for cyanide during a typical brei extraction procedure, using I. copper acetate—benzidine acetate reagent (4), and II. alloxan reagent (25) with microscopic examination for crystals of oxaluramide. The following table shows that the ether extraction for the removal of the free auxin is effective in extracting unreacted cyanide from the aqueous phase.

But whether or not traces of free CN^- remaining in the aqueous phase after free auxin removal react with IAc subsequently produced, or whether continued IAc production is inhibited by the initial treatment with cyanide, is immaterial to the con-

	Ether ^a		Brei	
	I	II	I	II
Free auxin extracts				
1st $\frac{1}{2}$ hr.	+++	+++		
2nd $\frac{1}{2}$ hr.	++	++		
3rd $\frac{1}{2}$ hr.	+	+	~	—
Precursor auxin extract				
1st 24 hr.	—	±	~	—

^a For assay, HCN removed from ether by shaking with *N* NaOH.

clusions the data permit us to draw: that rapid IAA formation only occurs at the time when appreciable IAc is present, and that the reduction of IAc to insignificant levels coincides with the time of a rapid drop in IAA formation.

Effect of Dimedon on Indoleacetic Acid Yields from Leaf Breis. As was previously shown (8), dimedon inactivates IAc. If IAA arises from IAc, inactivation of the latter should result in a lowered level of IAA. Leaf dispersions were, therefore, incubated with and without dimedon and the resultant levels of IAc and IAA then determined.

Leaf bases were cut into small pieces, mixed, and divided into four 30 g. portions. Each was dispersed in cyanide solution as usual, except that to 2 of the portions 20 mg. of dimedon in water were added during the blending. With several drops CHCl_3 as a preservative, one pair was incubated for 24 hr. and the other for 96 hr. before ether extraction. To ascertain whether dimedon had any effect on IAA *per se*, 2.8 γ IAA in 50 ml. cyanide solution were also incubated with and without dimedon.

The data given in Table II show that the presence of dimedon in the breis does lower the IAA yields, by a ratio of 1:3 at 24 hr. and 1:250 at 96 hr. As can be noted from the lower portion of the table, under similar conditions dimedon is without effect on pure IAA.

TABLE II

Effect of Dimedon on: A. Indoleacetic Acid Yields from Incubated Leaf Breis; B. Crystalline Indoleacetic Acid

	24 hr.	96 hr.
A. Leaf breis	γ IAA $\times 10^{-3}$	
With dimedon	11	9
Without dimedon	36	2300
B. IAA (2.8 γ)	γ IAA recovered	
With dimedon	—	2.3
Without dimedon	—	2.0

No IAc was obtained from the dispersions with or without dimedon either at 24 or at 96 hr. It was repeatedly observed that no IAc could be extracted from incubated leaf breis, prepared both with and without cyanide, unless ether was present during the incubation. If we assume that a rapid conversion of IAc to IAA occurs in the aqueous phase of a brei, then it is probable that the ether simply functions to extract and trap part of the aldehyde before it can be converted.

Conversion of Indoleacetaldehyde to Indoleacetic Acid by Leaf Tissues. Since the preceding experiments indicated the relation of IAA origin to IAc, the leaf tissues were then tested to determine whether they were able to convert added IAc to IAA. Purified preparations of IAc were incubated with leaf dispersions and with the crude enzyme preparation obtained from the leaf bases.

TABLE III

Conversion of Indoleacetaldehyde to Indoleacetic Acid by Leaf Breis and Leaf Enzyme
The indoleacetaldehyde untreated by brei or enzyme yielded no activity

	Blending medium	γ IAc added	γ IAA $\times 10^{-3}$
Brei	Water	0	5
Brei	Water	1.3	400
Brei	0.001 M NaCN	0	60
Brei	0.001 M NaCN	26	2400
Enzyme	—	0	4
Enzyme	—	6.0	350
Enzyme (boiled)	—	4.2	7

Fifty g. of the leaf bases were blended and the resultant dispersion divided in half. One part was placed in a flask containing the residue remaining from the evaporation of the ether from a purified preparation of IAc. Several drops of CHCl_3 were added and the brei was incubated for 24 hr. As a control, the remaining half of the dispersion was treated in an identical fashion, except that the IAc was omitted.

For the enzyme incubation, IAc was treated with the enzyme at a pH of 6.8 as detailed under general methods.

Table III shows that incubation of IAc with both the breis and the enzyme results in markedly increased IAA yields. These results indicate the presence of an enzyme system in the pineapple leaf which is capable of converting IAc to IAA.

The effect on IAA levels of infiltrating IAc into discs of intact tissues was also determined. Although a small increase of acid auxin was observed twice, we could not replicate these results. Probably one factor

was the difficulty of obtaining sufficient IAc to insure that a significant quantity of the aldehyde was infiltrated.

The frequent observation that tryptophan can serve as a precursor of auxin in plant tissues was already mentioned. Wildman, Ferri and Bonner (28) have shown that an enzymatic mechanism does exist in spinach leaves for this conversion. They have also found that increased auxin levels result on infiltration of spinach leaves with indolepyruvic acid (IPA). From this they concluded that IPA was the probable intermediate in the conversion of tryptophan to IAA, as suggested by Thimann (22) for *Rhizopus*. An alternative pathway can also be

TABLE IV
Conversion of Tryptophan to Indoleacetaldehyde and Indoleacetic Acid
Controls of tryptophan alone yielded no activity

	Tryptophan	pH	Incubation	$\gamma \times 10^{-3}$	
				IAc	IAA
	mg.		hr.		
Discs	0	6.8	4	71	800
Discs	2.25	6.8	4	630	1900
Brei	0	5.1	0.5	0	9
Brei	5	5.1	0.5	26	15
Brei	0	5.1	24 ^a	0	28
Brei	5	5.1	24 ^a	450	1200
Enzyme	0	6.8	4	0	3
Enzyme	10	6.8	4	120	24

^a Incubation under ether.

visualized for this reaction, involving tryptamine and indoleacetaldehyde as intermediates. Wildman *et al.*, however, failed to obtain conversion of tryptamine or IAc to IAA by infiltration of the former into spinach leaves, or by incubation of both compounds with enzyme preparations able to convert tryptophan.

We have shown that not only does the pineapple leaf contain IAc and IAA but also that leaf breis and leaf enzymes can convert IAc into IAA. In order to clarify whether this reaction could or could not function in the natural sequence of tryptophan degradation to IAA, tryptophan, tryptamine, and IPA were investigated with particular reference to their effects on IAc levels.

Conversion of Tryptophan. Following the procedures given under general methods, L-tryptophan (Eastman) was incubated with weak cyanide dispersions of leaf tissues, infiltrated into leaf discs, and incubated with the enzyme preparation. Although, under some conditions,

TABLE V

Conversion of Tryptamine into Indoleacetaldehyde and Indoleacetic Acid

Medium for tryptamine·HCl: phosphate buffer, pH 6.8

Medium for tryptamine base: water

	Tryptamine	Exp. I		Exp. II	
		$\gamma \times 10^{-3}$		$\gamma \times 10^{-3}$	
		IAc	IAA	IAc	IAA
Tryptamine·HCl	mg.				
Discs	0	78	430	130	1000
Discs	2.25	130	480	170	1500
—	2.25	0	0	0	0
Experimental increase		1.7×	1.1×	1.3×	1.5×
Tryptamine base					
Discs	0	53	52	82	75
Discs	2.25	150	210	710	170
—	2.25	26	0	76	0
Experimental increase		1.9×	4.0×	4.5×	2.3×
Tryptamine·HCl					
Enzyme	0	0	3	0	4
Enzyme	10	530	150	660	180
—	10	0	0	0	0

auxin arises readily from tryptophan alone (7), parallel controls of tryptophan here yielded no auxin. Hence, it is evident from the data in Table IV that either dispersions or discs of pineapple leaves, or the enzyme mixture obtained from the leaves, readily convert tryptophan to both IAc and IAA.

The auxin arising from the conversion of tryptophan by leaf discs has similar sensitivities to hot acid and alkali as synthetic IAA. When the acid fraction of an ether extract from tryptophan-infiltrated discs was subjected to acid and alkaline reflux for 1 hr. (using the method

TABLE VI

Conversion of Indolepyruvic Acid to Indoleacetaldehyde and Indoleacetic Acid

A. Leaf discs infiltrated with 0.19 mg. IPA, phosphate buffer, pH 5.0

	$\gamma \times 10^{-3}$			
	Exp. I		Exp. II	
	IAc	IAA	IAc	IAA
1. IPA alone	8	50	180 ^a	2100 ^a
2. Discs alone	18	300	34	270
3. 1 + 2	26	350	210	2400
4. Discs + IPA	1100	2400	290	3900
Experimental increase	42×	6.9×	1.4×	1.6×

B. Leaf enzyme incubated with IPA. The enzyme alone yielded no IAc and trace values of IAA at both pH levels

	IPA	pH	$\gamma \times 10^{-3}$	
			IAc	IAA
Enzyme	mg. 2	5.0	760 ^a	59,000 ^a
—	2	5.0	1,900 ^a	22,000 ^a
Enzyme	0.25	5.0	970 ^a	12,000 ^a
—	0.25	5.0	1,000 ^a	6,000 ^a
Enzyme	2.0	6.8	290	20,000
—	2.0	6.8	610	29,000
Enzyme	0.25	5.0	53	6,500
—	0.25	5.0	97	4,400

^a For individual determinations so designated, the acid and neutral fractions were separated by adjusting the pH of the buffer containing IPA to 8.0 and removing the IAc with ether. The aqueous phase was then brought to pH 2.8 and again partitioned with ether to obtain the acid auxin.

In the remaining units of these experiments, either the incubation mixtures or the discs were directly extracted with ether and the ether then partitioned with bicarbonate-glucose solutions for removal of the acid fraction.

described in (8)), complete inactivation in the acid resulted. (In γ IAA $\times 10^{-3}$, no reflux = 210, reflux in water = 140, reflux in acid = 0, reflux in alkali = 130.)

Conversion of Tryptamine. Tryptamine·HCl (Eastman) was then infiltrated into leaf discs and incubated with the enzyme preparation (Table V). Infiltration of the amine salt showed a small but repeatable increase in both IAc and IAA. Typical results are given in the table. It occurred to us that the small experimental increase noted might be due to a low cellular permeability to the hydrochloride, particularly in view of the results subsequently secured by enzyme incubation. Therefore, the free base was prepared by neutralization of the salt with an equivalent amount of alkali and crystallization of the resultant oil. When the tryptamine base was infiltrated, higher conversion ratios were obtained for both IAc and IAA. It is of interest to note that some IAc was obtained from the parallel run of tryptamine base without leaf tissue.

The results with the enzyme incubation are more striking in verifying the presence of a mechanism in the pineapple leaf for the transformation of tryptamine into IAc and IAA.

Conversion of Indolepyruvic Acid. Similarly, IPA was infiltrated into leaf discs and incubated with the enzyme preparation. The results of infiltration are given in Table VI-A. Although IPA spontaneously decomposes into both IAc and IAA under these conditions, the infiltrated leaf discs showed an increase in IAc of $42\times$, and a $6.9\times$ increase in IAA, indicating the presence in the leaf of an IPA conversion system.

These ratios of increase were reduced, due to high control values of IPA alone, when the pH of the IPA control in aqueous solution was first made alkaline and then acid to separate the neutral and acid fractions. Apparently, the acid or alkali (or both) accelerated the decomposition of IPA to IAc and IAA.

On incubation of IPA with the enzyme preparation (Table VI-B), a significant rise in IAA was obtained at a pH of 5.0; however, the IAA yield at the pH of 6.8, and the IAc levels at both pH 5.0 and 6.8 are lower than those arising from the control decomposition of IPA. Our results at pH 6.8 agree with the results of Wildman *et al.*, who found at this pH not only no increase but actually a decrease in *acid* auxin upon incubation of IPA with spinach enzyme. Nevertheless, we do obtain a rise in acid auxin or IAA, consistently repeatable, upon *in vitro* enzymatic incubation of IPA at a pH of 5.0. It would be of interest to ascertain the pH optimum of the IPA converting enzyme.

The decrease in levels of IAc upon incubation of IPA with the enzyme (Table VI-B) is difficult to interpret. It is as though part of the IAc formed by the spontaneous decomposition of IPA was converted to IAA without any *enzymatic* transformation of IPA to IAc occurring. One explanation suggested is that the leaf discs contain both the system capable of simultaneous "oxidative decarboxylation" (11) of IPA directly to IAA as well as a carboxylase catalyzing the reaction IPA to IAc, and that the latter enzyme was lost in the preparation of our enzyme mixture. But this explanation is incompatible with the observation that greatly increased amounts of IAc result when tryptophan is incubated with the same enzyme mixture, unless the major reaction with tryptophan is by way of tryptamine.

The data which indicate that one of the products of the spontaneous decomposition of IPA is IAc (Table VI), the significant rise in IAc level obtained by *in vivo* infiltration of IPA, the ability of the leaf to convert IAc to IAA, and the widespread occurrence of the carboxylase system in higher plants, make it more probable that IAc is an intermediate in the conversion of IPA to IAA by the leaf. However, additional evidence to clarify whether the conversion of IPA to IAc in the leaf is an enzymatic or spontaneous process would be desirable.

The Indoleacetic Acid Inactivating Enzyme in the Pineapple Leaf. It was frequently observed that, without the presence of cyanide in unboiled breis, low or erratic auxin yields were obtained. This suggested the presence of a cyanide-inhibited, auxin-inactivating enzyme in the pineapple, similar to that described by Tang and Bonner (21). Therefore, IAA was incubated with our enzyme mixture both with and without the addition of cyanide.

Two media were prepared containing 50 ml. 0.1 M $\text{KH}_2\text{—Na}_2\text{HPO}_4$ buffer, pH 6.5 100 γ IAA, and 1 ml. of the enzyme dispersion. In one were dissolved 25 mg. NaCN before addition of the enzyme, making the mixture 0.01 M NaCN. After incubation, for 4 hr. at 28°C., the digests were acidified to pH 2.8 and partitioned with ether to recover the IAA. From the incubation without cyanide, 41 γ IAA were obtained, while from the cyanide-treated digest 94 γ were recovered, an inactivation of 53 γ IAA. Still greater inactivation resulted in a duplicate experiment, while from a similar incubation of 100 γ IAA plus 1 ml. of boiled enzyme dispersion 88 γ IAA were recovered. Since IAA itself is insensitive to cyanide (28), these results show that, without cyanide, over 50% of the activity originally present was inactivated by the enzyme preparation.

It is, therefore, probable that some IAA inactivation took place in all of the enzyme incubations described in the preceding experiments. Hence, the concentrations of IAA listed as resulting from the incubation of IAc, tryptophan, tryptamine, and IPA with the enzyme preparation, may be considered as highly conservative values.

DISCUSSION

The experiments reported here substantiate the hypothesis that IAc is the immediate precursor of IAA. The data can be recapitulated as follows:

1. There is a high correlation between IAc levels and the amounts of IAA arising from the precursor of auxin in various tissues of the pineapple plant.

2. As shown by periodic extractions of leaf breis, rapid formation of IAA occurs as long as appreciable quantities of IAc are present, while a sharp drop in IAA yields occurs when IAc concentrations fall to negligible levels.

3. The presence of the aldehyde reagent dimedon in leaf breis reduces markedly the yields of IAA obtainable.

4. Incubation of IAc with either leaf breis, or the enzyme mixture prepared from the leaf, results in large increases in IAA levels, indicating the existence in the pineapple of a system able to convert IAc to IAA.

5. Increased yields of IAc, as well as IAA, are obtained when theoretical precursors of IAA are incubated with the leaf and/or with breis or enzymes prepared from the leaf.

Points 2, 3, and 4 may be considered as evidence against the likelihood that both IAc and IAA arise independently from a common precursor.

It would be of interest to determine whether the transformation of the aldehyde to the acid is an aldehydrase or a mutase action; if the latter, the simultaneous formation of tryptophol would be expected. Hemberg (10) did consider that possibly occurring tryptophol in his neutral fractions of potato extracts might be the source of IAc. However, he was unable to obtain any activity from tryptophol treated either by milk, or by alcohol dehydrogenase and then Schardinger enzyme.

Concerning the origin of IAc, we have shown that tryptophan is readily converted to IAc, as well as IAA, when infiltrated into leaf discs, added to leaf breis, or incubated with the leaf enzyme preparation. IPA or tryptamine would be the likely theoretical intermediates in a degradation of tryptophan to IAc. It has also been shown that the pineapple leaf can convert both of these compounds to the aldehyde, indicating that the formation of IAc in the leaf could take place with either tryptamine or IPA as intermediates. These reactions are represented in Fig. 3.

Whether the formation of IAc proceeds normally in the pineapple through IPA or tryptamine remains to be definitely ascertained. As Larsen (12) has pointed out, oxidative deamination of amino acids in the animal body is generally considered to take place with the intermediate formation of α -keto acids, and there is considerable evidence that this type of reaction also takes place in the amino acid metabolism of higher plants (3,14). On the other hand, extremely little is known about tryptamine metabolism in the plant. Small amounts of this amine are known to occur in plants, particularly in certain species of *Acacia* (27). The well-known bacterial decarboxylation of amino acids to yield the analogous amine apparently does not apply to tryptophan (5). For the animal body, however, some information is available. Thus, incubation of tryptophan with kidney extracts yields a

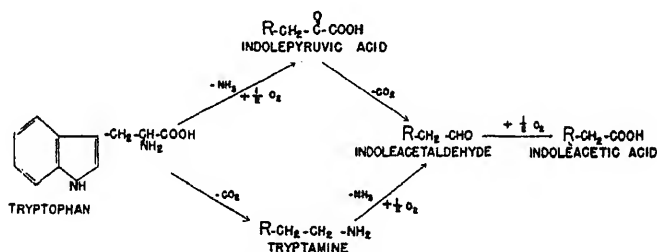


FIG. 3. Pathways of tryptophan conversion to auxin in the pineapple leaf.

substance with the characteristic pressor action of tryptamine (26). Tryptamine, as well as tryptophan and IPA, is partly converted to IAA by the liver and kidney of the dog (20), while an aldehyde and ammonia are formed upon tryptamine oxidation by the amine oxidase of brain tissues (17). These reactions fall into the pathways suggested in Fig. 3 for tryptophan conversion.

Our results with tryptophan and IPA are generally in accord with those of Wildman, Ferri and Bonner (28) insofar as the effect of these compounds on IAA level is concerned. However, the above workers could find no conversion of tryptamine or IAc by leaves of spinach or enzymatic preparations made therefrom. These differences may be due to the different plant materials used. It is pertinent to note that tryptamine is also transformed to auxin in the *Avena* coleoptile, giving a

strong but delayed auxin response (18). An explanation for the lack of agreement in our results with IAc may lie in the nature of the IAc preparations employed. It was not shown by Wildman *et al.* that their preparation was potentially active, *i.e.*, that it could be converted to IAA by such agents as soil or aldehyde systems, whereas we have used the regenerated bisulfite addition product from native neutral fractions, whose convertibility by Larsen's methods was demonstrated (8).

The applicability of the concept that the principal precursor of IAA is tryptophan to the natural formation of auxin in the plant has been discussed elsewhere (2,28). Conflicting evidence for considering tryptophan to be the primary source of *auxin* in tissues (as differentiated from tryptophan as the principal origin of IAA) was dealt with in a recent review by Skoog (19). We consider that the question as to whether tryptophan, auxin-protein complexes (6), or a "precursor" such as that isolated from the corn endosperm (1) function as the source of the native auxin in a plant tends to remain moot unless, 1) the identity of auxin in the particular organ dealt with is delimited; in terms of physiological function, the most desirable delimitation would be in the free auxin extract; 2) the precursor occurs naturally and produces the same auxin that is native, preferably at physiological temperatures and hydrogen ion concentrations; 3) the mechanism(s) responsible for conversion of the precursor into auxin is shown to be present.

No indications were obtained of any of the hitherto characterized native auxins other than IAA in free auxin extracts of the pineapple leaf (8). The present work has demonstrated the presence of the mechanisms for the conversion of tryptophan and theoretical intermediates to IAA, and has described experiments which indicate the direct relationship between IAA formation and IAc. We therefore believe that the tryptophan hypothesis offers the most satisfactory explanation for the origin of the native auxin in the pineapple leaf.

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SUMMARY

1. Additional evidence that the production of auxin from its precursor in the pineapple leaf is an enzymatic process, was obtained from incubations of boiled and unboiled tissue dispersions.

2. Both the pineapple leaf and a crude enzyme preparation obtained from the leaf are able to convert tryptophan into indoleacetaldehyde and indoleacetic acid.

3. It was shown that indoleacetic acid and indoleacetaldehyde can arise from tryptophan through either tryptamine or indolepyruvic acid as intermediates.

4. Data are presented which indicate that indoleacetaldehyde is the immediate precursor of indoleacetic acid in the pineapple plant.

5. There is also present in the pineapple leaf the cyanide-inhibited enzyme system which inactivates indoleacetic acid.

6. These experiments are interpreted as evidence in support of the concept that tryptophan is the origin of the native auxin in the pineapple leaf.

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Urea and Ammonia Content of Mouse Epidermis ¹

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INTRODUCTION

This is one of a series of studies dealing with the nitrogen metabolism of mouse epidermis in various phases of growth. It is necessary to characterize this tissue as completely as possible, since little or no data are available on the nitrogen metabolism of the epidermis of the mouse, or of any other species. The rapidity with which repair takes place in the epidermis, after injury in the healthy organism, suggests that this tissue possesses a remarkable capacity for the synthesis of proteins. Urea and ammonia were chosen as the first nitrogenous constituents to be studied, because unusually high values were encountered for these substances in experiments in which the arginase activity was being measured. The nature of the changes which were observed in the ammonia and urea contents during post-natal development suggested that these two constituents might have a close metabolic relationship in epidermis.

EXPERIMENTAL

Materials and Methods

Samples of epidermis were rapidly removed from the dermis at 50°C. on a constant temperature hot plate, weighed rapidly on a torsion balance, and then immediately immersed in approximately 2 ml. of redistilled water contained in the calibrated tubes of 5 ml. long-stemmed ground-glass homogenizers which had been previously placed in a beaker of gently boiling water. The same procedure was employed for other tissues studied. The tube was removed after 10 min., cooled, the fluid brought to a suitable volume, and the tissue homogenized. There was no indication of an increase in the free ammonia content during the preparation of tissues. Homogenates were employed for the determination of urea and preformed ammonia by the procedures recommended by Conway for use with microdiffusion units (2), except that the incubations with urease were carried out for 1 hr. in test tubes, and aliquots of the

¹ Aided by grants from the U. S. Public Health Service and the Charles F. Kettering Foundation.

incubation mixture were analyzed for ammonia content. Suitable blanks, and the values for preformed ammonia, were subtracted in calculating the urea content. Boric acid, containing the mixed indicator of bromocresol green and methyl red, was employed as absorbent. The titrations were performed with 0.02–0.03 *N* HCl delivered from a micrometer buret, the smallest division of which corresponds to 3.16×10^{-4} ml. Quantitative recoveries were achieved both from pure solutions and from tissue homogenates to which ammonia and urea had been added singly or together. Excellent agreement was found among replicate analyses of the same homogenate, and the ammonia and urea contents were shown to be proportional to the quantity of tissue in aliquots from a given sample. It is considered probable that the preformed ammonia exists in the tissue in the form of ammonium salts, and possibly as amides which are labile under the conditions employed.

Adult female Swiss mice were painted with reagent grade benzene and with 0.1% croton oil thrice weekly. In most instances the experimental mice were sacrificed 5 days after the last application of the test solution, and the control mice 5 days after the backs were shaved. The methods of painting and the removal of the epidermis from the dermis were the same as those previously described (1,3). Exceptions to these procedures will be noted in the description of the individual experiments.

It was not necessary to shave any of the baby mice prior to the 9th day after birth to obtain satisfactory samples of epidermis. Mice between the 9th and 25th days of age were sacrificed either on the day on which they were shaved or on the morning after. It was not possible to obtain suitable tissue samples from embryos at periods earlier than 3 days prior to birth.

RESULTS

Comparison of Urea and Ammonia Contents of Epidermis and Dermis (Table I)

There is no contamination of the epidermis with dermis when the tissues are separated by heat (1). Nevertheless, it seemed desirable to

TABLE I
Comparison of Urea and Ammonia Contents of Epidermis and Dermis
Results expressed in mg. N/100 g. fresh tissue

Age	Number of mice	Urea-N		Ammonia-N	
		Epidermis	Dermis	Epidermis	Dermis
		mg.-%	mg.-%	mg.-%	mg.-%
9–12 Months	3	45	24	22	19
9–12 Months	3	87	24	24	20
9–12 Months	3	77	20	21	17
7 Days	2	111	28	95	29
7 Days	2	131	40	106	20

determine whether the extraordinarily high contents of urea and ammonia in mouse epidermis could possibly result from a contamination of the latter tissue with minute amounts of dermis. In Table I are shown results obtained from tissues of animals widely separated in age, and differing in the content of the constituents studied. In every case, the contents of ammonia and urea in the epidermis were higher than in the dermis. It can, therefore, be concluded that the high levels of these substances observed in epidermis are genuinely characteristic of that tissue.

*Comparison of Mouse Epidermis with Mouse Liver, Kidney
and Blood and with Human Epidermis*

The data in Table II show that mouse epidermis contains significantly higher concentrations of urea and ammonia than do kidney, liver, and blood, and a greater urea content than human epidermis. The ammonia values recorded for human epidermis may be somewhat too high because of the necessary delay between the removal of the tissue and the time it became available for analysis. It is interesting

TABLE II

*Comparison of Mouse Epidermis with Mouse Kidney, Liver, and Blood
and with Human Epidermis*

All values for tissues expressed as mg. N/100 g. fresh tissue;
values for blood as mg./100 ml.

Tissue	Number of determinations	Urea-N		Ammonia-N	
		mg.-%			
		Mean	Range	Mean	Range
Mouse epidermis	20	77	(55-98)	24	(16-31)
Human epidermis ^a	6	33	(17-64)	24	(17-46)
Mouse kidney	3	35	(30-39)	17	(14-20)
Mouse liver	7	21	(15-30)	11	(7-15)
Mouse blood	10	16	(13-21)	<i>b</i>	—

^a Epidermis obtained from normal areas of skin taken from the pectoral area as part of radical mastectomies.

^b Ammonia values are not recorded for the blood specimens because the special precautions necessary for accuracy could not be maintained. The values actually obtained for use as "blanks" in the urea determinations averaged 3 mg.-%.

that the samples of human epidermis which were studied had a lower mean value for urea than did mouse epidermis, even after treatment with croton oil (see Table V).

Changes with Age in Epidermal Urea and Ammonia

The ammonia nitrogen content of mouse epidermis (Table III) increased regularly from the low mean value of 13 mg.-% 2-3 days before birth to 104 mg.-% on the 5th day after birth. There was little or no

TABLE III
Changes with Age in Epidermal Urea and Ammonia of Normal Mice
Results expressed in mg. N/100 g. fresh tissue.

Age	Number of determinations	Number of mice	Urea-N		Ammonia-N	
			mg.-%			
			Mean	Range	Mean	Range
2-3 days prenatal	4	12	22	(20-25)	13	(10-17)
1 day prenatal	8	14	55	(42-68)	22	(17-28)
2 hr. old	3	9	59	(49-66)	36	(32-40)
1.5 days old	7	14	47	(33-68)	68	(60-79)
3 days old	5	18	68	(63-69)	83	(71-95)
4 days old	4	12	88	(74-109)	94	(86-102)
5 days old	4	12	120	(114-129)	104	(100-110)
6 days old	2	6	120	(117-121)	109	(106-110)
7 days old	4	9	116	(107-131)	106	(95-115)
8 days old	3	10	146	(141-149)	129	(107-142)
9 days old	1	5	144	—	160	—
11 days old	3	9	81	(76-89)	50	(47-55)
12 days old	5	20	73	(67-77)	45	(38-54)
20 days old	1	6	100	—	28	—
21 days old	1	13	99	—	27	—
24 days old	1	8	101	—	33	—
2.5-4.0 mos. old	20	92	77	(55-98)	24	(16-31)
9-12 mos. old	7	26	63	(37-97)	26	(21-37)
20 mos. old	1	1	56	—	22	—

change for the next 2 days, but the values rose again on the 8th day and attained the peak value of 160 mg.-% by the 9th day. On the 11th day a highly significant decrease to 50 mg.-% had occurred, with a further decrease being registered on the 20th day. The values thereafter were

relatively constant, with little or no change being found in the remaining age groups studied. The urea nitrogen values also began at the very low levels of 22 mg.-% in the earliest group studied. The changes were somewhat irregular until 1.5 days after birth. The values increased from the 2nd to the 5th day after birth, at which time the level of urea was 120 mg.-%, and then remained virtually constant through the 7th day. The maximal values of 146 and 144 mg.-% were found on the 8th and 9th days, respectively. There was a decrease to 81 mg.-% on the 11th day, and to 73 mg.-% on the 12th day, an increase to approximately 100 mg.-% on the 20th to 24th days, and a drop in the remaining age groups studied. It is interesting that both the urea and ammonia

TABLE IV
Changes with Age in Epidermal Urea and Ammonia in the Rat
Results expressed in mg. N/100 g. fresh tissue.

Age	Number of determinations	Number of rats	Urea-N		Ammonia-N	
			mg.-%			
			Mean	Range	Mean	Range
12 days	1	1	208	—	103	—
6 weeks ^a	1	1	101	—	90	—
6 months ^a	2	2	41	(38-45)	27	(26-28)
2 years ^a	4	4	59	(44-77)	36	(30-45)

^a Approximate ages.

showed rises after birth, and that both attained the maximal values on the 8th and 9th days. The values for ammonia nitrogen were approximately equal to those found for urea nitrogen between the 2nd and 9th day after birth. Thereafter, the values for urea nitrogen were uniformly higher than for ammonia nitrogen. This indicates that any relationship existing between them may have undergone a change between the 9th and 11th days after birth. The values for urea and ammonia in the epidermis of the 20 month old mouse were virtually identical with those obtained for embryos one day before delivery.

Table IV contains data for rats which indicate that changes in urea and ammonia content may take place in the epidermis of the rat in a fashion similar to that described above for the mouse.

*Influence of Benzene Alone and Benzene Containing 0.1% Croton oil
on Epidermal Urea and Ammonia (Table V)*

Three paintings with benzene alone, which causes a mild hyperplasia, produced an increase in the urea content and no change in ammonia content. Six paintings caused a decrease in urea and an increase in ammonia. The application of a dilute solution of croton oil in benzene

TABLE V
*Influence of Benzene Alone and Benzene Containing 0.1% Croton Oil
on Epidermal Urea and Ammonia*
Results expressed in mg. N/100 g. fresh tissue

Tissue	Number of deter- minations	Number of mice	Urea-N		Ammonia-N	
			mg.-%			
			Mean	Range	Mean	Range
Normal epidermis	20	92	77	(55-98)	24	(16-31)
3 paintings with benzene	6	22	116	(85-145)	25	(19-31)
6 paintings with benzene	16	42	54	(33-69)	35	(21-57)
1 painting with croton oil (5) ^a	10	25	71	(37-103)	29	(25-33)
6 paintings with croton oil (7) ^b	6	22	50	(32-62)	21	(14-26)
1 painting with croton oil (10) ^a	5	10	46	(42-53)	21	(16-25)
6 paintings with croton oil (17) ^a	10	22	42	(27-65)	36	(32-47)

^a Number in parentheses refers to the number of days between the first application and the time the analyses were performed.

^b Solution applied on 6 successive days and the animals were killed on the 7th day after the first application.

produces a very marked hyperplasia which regresses after cessation of treatment and which does not result in the destruction of sebaceous glands or in tumor formation.² The results obtained with croton oil in benzene are listed according to the number of days between the first application and the time of analysis of the tissue rather than according to the number of applications of the solution, since the decrease in urea which was observed was better correlated with the former. Croton oil in benzene produced a progressive decrease in urea content, but the changes in ammonia content were of doubtful significance, with the

² Berenblum, personal communication, and unpublished observations from this laboratory.

exception of the increase of ammonia observed in the group receiving 6 paintings of croton oil solution over a period of 17 days. This increase was comparable to that found when benzene alone was applied in a similar manner.

DISCUSSION

Epidermis of the normal adult mouse has been found to contain extremely high concentrations of urea and preformed ammonia. Even higher levels of these substances were observed in epidermis of mice during the first 9 days of post-natal development. Maximal values for urea and ammonia were attained at 9 days, and were 2 and 8 times higher, respectively, than those found in the adult tissue. Such high concentrations would be expected in tissues which either form or concentrate these substances. The parallelism in the quantitative changes in urea and ammonia in the early stages of development suggests that an intimate relation exists between them in epidermis. Experiments are now under way to determine whether epidermis can synthesize urea from ammonia by the ornithine cycle, whether urea can give rise to ammonia and carbon dioxide in epidermis by urease action, and whether epidermis can concentrate urea from the blood. There is no known mechanism other than diffusion into the blood by which the avascular epidermis of the mouse, which contains no sweat glands, can excrete the urea.

It has been shown that the urea and ammonia contents of mouse epidermis can be altered by treatment with benzene or with a dilute solution of croton oil in benzene. However, elucidation of the physiological meaning of the results reported herein must await further characterization of the nitrogen metabolism of this tissue.

SUMMARY

1. Epidermis of normal adult mice was found to have higher concentrations of urea and preformed ammonia than the dermis, liver, kidney, or blood, and a greater urea content than human epidermis.
2. Even higher levels of urea and ammonia were observed in epidermis of mice during the first 9 days of post-natal development, the maximal values attained for urea and ammonia being 2 and 8 times higher, respectively, than those found in the adult animals.

3. The application to the skin of mice of pure benzene, which produces a very mild hyperplasia, or of 0.1% croton oil in benzene, which produces a marked hyperplasia, resulted in changes in the urea and ammonia contents of the epidermis.

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The Occurrence of Pentose- and Phosphorus-Containing Complexes in the Urine of Patients with Progressive Muscular Dystrophy¹

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INTRODUCTION

The purpose of this paper is to present (a) evidence indicating the constant presence of pentose-containing complexes in the urine of patients with progressive muscular dystrophy and (b) data compatible with the hypothesis that the material excreted is a ribose nucleotide which readily disintegrates to form cleavage products, which serve as an index to the composition of the original complex.

EXPERIMENTAL

The investigation was initiated by the repeated observation that positive reduction tests (to the degree ordinarily read as 1+) were obtained whenever 8-10 drops of such urine were heated for 30-40 min. in a boiling water bath with 5 cc. of Benedict's qualitative sugar reagent. Our interest was to find out what constituent of the urine was responsible for this so constant finding.

Our first approach was to eliminate as completely as possible the normal constituents which might contribute to the reducing capacity of urine. Fermentable sugars could, of course, be eliminated by yeast. Folin and Berglund (1) have shown that most of the creatine, creatinine and uric acid can be removed by Lloyd's reagent. The possibility, therefore, that any of these several constituents was the principal reducing agent was ruled out by the fact that positive tests persisted after treatment of these urines with yeast suspension and Lloyd's reagent, according to the method of Van Slyke and Hawkins (2). Qualitative tests for furfural were positive, a finding equally compatible with the presence of pentose or glycuronates either of which might cause reduction of alkaline copper sulphate. Benedict and Osterberg (3) have described a

¹ Part of this work was aided by a grant from the John and Mary Markle Foundation.

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method for the removal of glycuronates, polyphenols and various other substances from the urine by treatment with mercuric nitrate. Solutions of simple pentoses or hexoses can be carried through this procedure without loss. When applied to the study of urine obtained from patients with dystrophy, quite different results were obtained, depending upon whether freshly voided specimens of urine were immediately treated with mercuric nitrate or were preserved with thymol and kept in the laboratory for several days before being subjected to the same treatment. The reducing substance was removed from the fresh specimens but escaped precipitation after aliquots of the same sample of urine had been allowed to "age" for several days. Thus, it appeared probable that the reducing agent was a pentose which was being lost from the body as a compound which could be precipitated by mercury but which gradually disintegrated to release free pentose. In accord with this concept was the repeated observation that, with the "aged" specimens, the reduction of Benedict's solution occurred more promptly than the interval of 30-40 min. required by freshly voided urines to produce maximum reduction.

The pentose complexes which seemed most likely to be encountered were nucleotides or nucleosides originating from nucleic acids or from such compounds as adenosinetriphosphate or the various coenzymes which contain pent-nucleotides. The pentose of thymus nucleic acid has been shown by Levene (4,5) to be D-2-ribodeseose. More recently, other nucleic acids of animal origin have been shown to contain D-ribose (6). The pentose present in adenosinetriphosphate is D-ribose (7) and this same sugar is present in certain coenzymes. The desoxypentose of thymic nucleic acid forms no osazone with phenylhydrazine but ribose readily forms an osazone for which melting points varying from 156 to 162°C. have been reported.

From the urines of patients with muscular dystrophy a small yield of osazone was readily obtained in the following manner. The sample of urine to be tested was preferably preserved with thymol and "aged" for a few days. It was then treated with yeast suspension and Lloyd's reagent to remove any traces of glucose which might be present. Any desired volume of this treated urine was then mixed with approximately one-half its volume of a freshly prepared solution containing 5% phenylhydrazine hydrochloride, 20% sodium acetate and 10% acetic acid. The mixture was heated for 1 hr. in a boiling water bath. The heat was then turned off and the tubes or flasks allowed to remain in the bath to cool slowly and to stand for several hours. A small amount of brownish yellow sediment settled out, which, on microscopic examination, usually showed clear-cut crystalline osazones intermingled with varying amounts of amorphous debris. Occasionally, if the yield of osazone was very small, the crystals were missed in this first impure sediment but became readily distinguishable after recrystallization.

After trying numerous less satisfactory procedures we were finally successful in separating the osazone from other material in the following manner. The first crude sediment was removed by filtration and dissolved in 95% alcohol. The resulting dark brown solution was again filtered and evaporated to dryness in a small porcelain dish on a steam bath. The brown amorphous residue was then extracted repeatedly with small amounts of boiling water, which dissolved the osazone and left most of the brown waxy material adhering to the dish. The hot aqueous extract was filtered into a test tube immersed in hot water and allowed to remain there to cool slowly. From this

solution a small yield of an unmistakable yellow crystalline osazone was obtained contaminated with only a small amount of persisting debris. Several such crops of partially purified osazones were pooled and subjected to 4 more recrystallizations carried out as described above. This yielded clean crystals uncontaminated by any visible foreign material. The size and pattern of the crystals obtained varied considerably depending upon the amount present, the state of purification, and the rate of cooling. The most characteristic form, however, was that of a shiny, sharp, somewhat flattened yellow needle, which tended to form rosettes with the needles radiating in several planes from a central point. The repeatedly recrystallized osazones were filtered from the aqueous solution, washed with ice cold distilled water and dried. The melting point was 156.5–158°C. A ribosazone prepared in the same manner from ribose derived from yeast nucleic acid melted at 156.5–157°C., and a mixture of the two osazones melted at 156.5°C. On chemical analysis, the osazone prepared from urine gave the following results, which agree reasonably well with the calculated analysis of pentosazone: Found—C, 61.92%; N, 16.92%; H, 6.13%; O (by difference), 15.03%. Calculated—C, 62.53%; N, 17.07%; H, 6.14%; O, 14.26%.

The same osazone was also prepared from filtrates obtained following the treatment of "aged" urines with mercuric nitrate as described earlier.

With so much evidence that we were dealing with ribose which was being excreted as an organic complex it seemed desirable to determine whether these urines also contained nucleotide phosphorus. It was obvious that such studies would have to be done on freshly voided urine which had been handled with every precaution to retard the now recognized spontaneous disintegration of whatever pentose complex might be present. Specimens were, therefore, obtained at the bedside, where the patient voided directly into a bottle which had already been immersed in a mixture of salt and ice. The urine was then kept in a semifrozen state while it was brought to the laboratory where chemical studies were promptly started.

Three types of phosphorus studies were carried out, which included determination of (a) pre-existing inorganic phosphate in fresh urine, (b) inorganic phosphate following 7 min. hydrolysis with 1 *N* HCl, and (c) total inorganic phosphate following wet ashing of the urine with HNO₃ and H₂SO₄. The colorimetric method of Lowry and Lopez (8) was employed, as it offered a means of determining pre-existing inorganic phosphate at a pH. which would result in a minimum of hydrolysis of labile phosphate complexes which might also be present. The aliquots of urine submitted to 7 min. hydrolysis and to acid digestion were subsequently diluted and neutralized to the appropriate pH, and the inorganic phosphate measured by the same colorimetric procedure. The color densities were determined with a Klett-Summerson photoelectric colorimeter and the values for phosphate calculated on the basis of readings obtained with standard solutions of inorganic phosphate.

The data presented in Table I indicate the presence of organically bound phosphate in the urine of 3 patients with progressive muscular dystrophy. A considerable proportion of this bound phosphate was released by 7 min. hydrolysis with 1 *N* HCl which is the generally accepted criterion for the easily hydrolyzable phosphate associated with pyrophosphate linkages such as occur in adenosinetriphosphate. Our data contain no proof that all the ribose present is excreted as ribonucleotides or that it necessarily has a common origin with the organically bound phosphate. Rather strong

TABLE I
Partition of Total Phosphorus in Freshly Voided Urine

Source of urine	Date	Preexisting inorganic P	Inorganic P after 7 min. hydrolysis with 1 N HCl	Total P	Remarks
	1946	mg.-%	mg.-%	mg.-%	
H. J.	5/16	16.0	33.0	39.2	Patient with advanced typical muscular dystrophy
H. J.	5/28	46.0	52.9	67.5	
H. J.	5/29	65.0	78.8	102.0	
H. J.	5/30	69.0	77.0	85.0	
H. J.	6/17	56.2	63.3	75.6	
H. H.	6/25	5.4	6.4	11.0	Typical P. M. D. for several years
G. K.	12/17	92.0	108.0	114.0	
D. D.	5/29	25.0	—	26.0	Moderately severe dystrophy
D. D.	6/25	37.0	36.8	36.4	Normal person
M. G.	6/25	165.0	163.5	163.0	Normal person

circumstantial evidence that this is the case is, however, afforded by the repeated observation that, on "aging" these urines, there was a simultaneous appearance of free pentose and disappearance of so-called 7 min. phosphorus. The phosphate fractions were investigated in numerous specimens of urine from normal individuals and in no case was there any appreciable difference in the values obtained for total phosphate and the pre-existing inorganic phosphate.

COMMENT

The significance of the excretion of ribose-containing complexes in the urine in progressive muscular dystrophy is not yet clear. We have thus far had the opportunity to study the urines from more than 20 typical cases and, in many instances, have observed them repeatedly over long periods of time. In no instance have we failed to demonstrate the presence of pentose complexes by the qualitative tests and osazone formation under the conditions we have outlined. Equally painstaking studies have given negative results in normal urines and in urine from chronically ill bed-ridden patients without muscular disease.

Loofbourow and his associates (9) have demonstrated the release of nucleotides and nucleosides from damaged living cells into their surrounding medium. Di Marco (10) has reported the presence of increased nucleotide nitrogen in the blood of patients with progressive muscular dystrophy. If such material finds its way into the blood stream, such

amounts as escape disintegration in the blood stream or urinary bladder would probably appear in the freshly voided urine. Calvery (11) has isolated small amounts of adenosine from large volumes (80 l.) of mixed human urine. It is possible that the constant excretion of these substances in muscular dystrophy is merely secondary to their release from disintegrating muscle cells. One observation, which we have had no opportunity to repeat, invites speculation in regard to a more primary significance. In this one instance pentose-containing complexes were detected in the urine of an apparently normal 3 year old brother of one of our patients with muscular dystrophy. There was a familial history of the disease, as one older brother of our patient and a maternal uncle had already died after running a typical course. Within the next 2 years this younger brother developed the typical clinical picture of rapidly progressing muscular dystrophy. It is possible that disintegration of muscle cells was already in progress before the appearance of detectable dysfunction. It is also conceivable that some inborn anomaly of vitally important nucleotide metabolism is responsible for the deterioration seen in clinical dystrophy.

It is our hope that an extension of these studies to include a greater variety of clinical conditions may help to evaluate the significance of the abnormal urinary findings we have reported. In view of Loofbourow's (9) observations, it appears that conditions which entail various types of acute injury to masses of cells should be investigated as well as various types of muscular disease. Such work is in progress and will be reported in another paper. Thus far, in a group of patients representing a wide variety of chronic muscular and neuromuscular disorders, pentose complexes have been found in the urine only in those conditions where the primary pathology is in the muscles.

SUMMARY AND CONCLUSIONS

The excretion in the urine of pentose-containing complexes has been shown to be a constant finding in patients with progressive muscular dystrophy.

Evidence has been presented which indicates that the pentose is ribose.

The presence of organically bound phosphate, part of which is hydrolyzable by 7 min. heating with 1 *N* HCl, has also been demonstrated in freshly voided urines from these patients. Thus, it is probable

that the pentose is in combination with phosphate and that the complex is either a ribonucleotide or some compound containing such a nucleotide.

The constant excretion of this complex in detectable amounts appears thus far to be a specific characteristic of patients with primary muscle diseases.

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Studies on the Carotenoid Pigments of *Neurospora*.

I. Composition of the Pigment ¹

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INTRODUCTION

This report deals with the identification and characterization of the carotenoid pigments comprising the orange coloring matter of *Neurospora crassa* (wild type). Interest in the *Neurospora* pigment stems from the early observations of Went (26) and van Deventer (4). Both workers noted that cultures of the mold grown in the dark were colorless, but that these cultures turned a bright orange when exposed to light. More recently, in the course of the extended investigations on *Neurospora* initiated by Beadle and Tatum (*cf.* 1), a variety of pigment mutants have been isolated, some of which have been shown to differ from the wild type by a single gene change (8). To investigate further the effect of light and gene mutation on pigment synthesis in this organism, a full knowledge of the composition of the pigment is required. It has been with this viewpoint in mind that the present investigation was undertaken.

The carotenoid nature of the *Neurospora* pigment was established by Went (26), van Wisselingh (28), and van Deventer (4). van Deventer further showed that the pigment of *Neurospora sitophila* included at least 3 components, one of which was tentatively identified as lycopene, $C_{40}H_{56}$. Crystalline preparations of the pigments were not obtained. It seems clear that at least one of the pigment fractions was not homogeneous, and the possibility exists, therefore, that the pigment of *N. sitophila* contains more than 3 components as claimed. This certainly is true for the coloring matter of the closely related species, *N. crassa*, as will be apparent shortly. However, it is not known to what extent the results are applicable to *N. sitophila*.

¹ This paper is based upon a part of a Dissertation presented to Stanford University. An abstract report has appeared elsewhere (7).

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The occurrence in *N. crassa* of phytofluene, a colorless C_{40} -polyene, has been reported previously (33). It was also shown that, whereas the biosynthesis of colored polyenes in *Neurospora* was markedly stimulated by continuous illumination during the growth period, the absolute amounts of phytofluene present were practically independent of such illumination.

EXPERIMENTAL

I. Materials and Methods

Technical grade solvents were employed in the extraction and chromatographic procedures. The petroleum ether was Skellysolve (b.p. 30–60°C. and b.p. 60–70°C.). The solvents used for spectroscopic and crystallization purposes, other than petroleum ether, were of reagent grade. Hexane was purified by filtration through silica gel, according to unpublished method of Polgár, to permit absorption measurements down to 220 $m\mu$.

The principal adsorbent used was $Ca(OH)_2$ (Sierra Brand Lime, ca. 300 mesh) mixed with a filter aid (Celite, 25% by weight). When rechromatographing the more strongly adsorbed pigments, a 2:1 mixture of $CaCO_3$ (Merck, heavy powder) and hydroxide was used. A 4:1 mixture of $Ca(OH)_2$ and alumina (Alorco, Grade F, –80 mesh) was frequently employed in the chromatography of the weakly adsorbed polyenes. Chromatographic adsorption tubes were those described by Zechmeister and Cholnoky (32).

Pigment solutions in polar solvents were transferred to petroleum ether or benzene with water and washed in a continuous-flow device, as described by LeRosen (14). The solutions were dried with anhydrous Na_2SO_4 . Pigment solutions were concentrated in an all-glass still under reduced pressure and a slow stream of CO_2 . Solutions were stored under CO_2 in a dark, cold room. Saponifications were carried out by treating petroleum ether solutions overnight at room temperature with an equal volume of 20% KOH in methanol.

Visual absorption maxima were determined with a Zeiss Evaluating Grating Spectroscope; usually, each pigment was tested before and after iodine catalysis [cf. Zechmeister (30)]. The values reported for the all-*trans* pigments represent the average (to the closest 0.5 $m\mu$) of several determinations.

A Beckman Spectrophotometer was employed in the determination of the absorption curves. Transmission measurements were made every 5 $m\mu$ except in the region of the maxima and minima, where the intervals were at 1 or 2 $m\mu$. Absorption curves of the isomerized pigments were made with iodine-catalyzed solutions (1% of the pigment) which had been illuminated by two Mazda fluorescent lamps (tube length 120 cm.) at a distance of 60 cm. for 20–30 min. In the case of the quantitative extinction curves the solutions were prepared by dissolving a little more than 1 mg. of crystals in 50 ml. hexane and making a 10-fold dilution of an aliquot sample for immediate determination. The extinction coefficients reported for neurosporene were calculated according to the formula, $E_{1\text{ cm}}^{1\%} = 1/Lc \log I_0/I$, where L = thickness of the stratum in cm., c = concentration in g./100 ml. solution, I_0 = incident light, and I = transmitted light. Qualitative extinction curves are presented as extinction plotted against wave length.

For crystallization purposes all solutions of a given pigment (obtained from various isolations) were combined, saponified, and rechromatographed until free of colored impurities. Unless otherwise stated, the crystallizations were effected by diluting a concentrated benzene solution of the pigment with absolute methanol. Usually 2 recrystallizations were carried out. In some instances, colorless impurities were encountered in certain pigment fractions, and, because the quantity of available pigment was small, pure preparations were difficult to obtain. Such impurities could frequently be removed by extracting the crude precipitate with hot methanol (*cf.* 37).

Crystalline preparations were dried in an Abderhalden apparatus *in vacuo* at 56°C. and stored in sealed tubes under CO₂. The melting points are corrected and were determined in a Berl block.

II. Cultivation of *Neurospora*

The wild type strain of *N. crassa* employed is designated by Beadle and Tatum (1) as 1A. The growth form and pigmentation of this strain are typical of all wild type strains;³ moreover, many of the existing color mutants were derived from it by treatment with mutagenic agents.

One of the morphological, nonconidiating mutants of *N. crassa*, No. 5801, was also cultured on a large scale to obtain a supply of dried mycelia sufficient for isolating crystallizable pigments. Qualitatively, no difference was noted in the pigments produced by the mutant and wild type. The major pigments obtained by extraction of some 500 g. (dry weight) of this mutant were combined, for purposes of crystallization, with the corresponding fractions isolated from wild type.

Several mass culture methods were investigated. Cultivation of the mold in Fernbach flasks partially filled with a liquid medium proved unsatisfactory, pigment formation being limited to that portion of the mycelial growth which was exposed at the liquid surface or extended above it. Although the growth was vigorous in submerged, well-aerated, liquid cultures, not all cultures produced pigment. Growth and pigmentation were uniformly good on a solidified agar medium and, in spite of the occasional difficulty encountered in freeing the mycelium from the agar, this method was adopted for mass culturing. The mold was grown on a modified, "complete" *Neurospora* medium (*cf.* 1). The basic salt solution was Fries No. 3 modified to contain only nitrate nitrogen. To this were added: sucrose, 5%; yeast extract, 0.5%; yeast autolyzate, 2%; malt extract, 0.5%; and agar, 1.5%. In contrast to the usual complete medium, the enriched medium appeared to support a denser and more intensely pigmented growth of mycelium. Loosely stoppered, 3 l. Fernbach flasks were used as culture vessels, each being filled with 400–500 ml. of the culture medium.

The cultures were incubated for 10–15 days in a constant temperature room. Its temperature varied during different runs but fell within a range of 29–36°C. Ordinarily, the cultures were maintained in darkness for 5–7 days and exposed to light

³ It may be noted that the pigment of *Neurospora* is contained in oil droplets or lipid granules within the cytoplasm. This is especially apparent in some of the morphological mutants where the hyphae are enlarged and abundantly filled with orange-colored droplets.

during the remainder of the incubation period. Where temperature control was not of major concern, as was the case with the extended mass culturing of the mutant, illumination was provided by incandescent lamps (usually 300 watts) placed at intervals about 40 cm. over the flasks. In the case of wild type, a more rigorous temperature control was made possible by using "Mazda Daylite" fluorescent lamps. The position of the flasks was changed daily to insure a more or less uniform light exposure.

In harvesting the mold, the agar substrate was cut into strips and removed from the culture vessels. The thick mycelial mats were peeled from the surface, and adhering bits of agar carefully scraped off with a dull knife. In the case of the mutant, the harvested growth was spread out on paper, air-dried at 30°C., and stored in the dark. Prior to extraction, this dry material was ground in a powder mill.

Only fresh mold was used for the pigment analyses of wild type. The freshly harvested growth (mycelia and conidia) was first coarsely fragmented by passage through a meat grinder and the resulting orange pulp extracted as described below. When quantitative estimations of the pigments were to be made, the dry weight of the harvest was determined on the basis of an aliquot sample. The calculated dry weight yield of wild type varied between 3-4 g. per culture flask.

III. Extraction of the Polyenes

The extraction from fresh material was carried out as follows (the preliminary dehydration was omitted when working with dried mold). The fresh mold (in the form of the pulp mentioned) was treated with 1-2 volumes of acetone for 12-18 hr. The colorless liquid was filtered off and the residue washed with fresh portions of acetone. When the last washings became colored, indicating that the dehydration was essentially complete, the residue was distributed in glass-stoppered bottles and shaken mechanically for 1 hr. in the presence of 1 or 2 volumes of acetone. The acetone, now deeply colored, was filtered off and the extraction repeated several times with fresh solvent. It was usually necessary to grind the residue under acetone in the presence of quartz sand. It was further treated with acetone until the extracts were only faintly colored and the residue was practically colorless.

All colored extracts were combined in a separatory funnel and the pigment transferred to petroleum ether by the cautious addition of excess water. In order to complete the transfer it was necessary to acidify the aqueous acetone (with acetic acid) and extract repeatedly with petroleum ether. The petroleum ether solutions were washed, concentrated to 100-200 ml., and saponified.

The pigment was then resolved by partition methods into its epiphasic and hypophasic constituents. To this end the mixture was placed in a separatory funnel and the colored, alkaline-methanol phase diluted cautiously with water. This step usually effected the transfer of all epiphasic pigment to the upper phase and the major portion of the hypophasic pigment could be drawn off. Some of the hypophasic pigment, however, remained in the petroleum ether. Repeated shaking of the latter with equal volumes of a 5% solution of KOH in 90% methanol served to complete the separation. The various alkaline methanol extracts were combined and the hypophasic pigment transferred back to petroleum ether by acidification and dilution in the manner men-

tioned previously.⁴ The two pigment fractions, each in petroleum ether, were washed, dried, and concentrated to a small volume.⁵

IV. Resolution of the Pigment Mixture and Characterization of the Components

The epiphasic and hypophasic pigment fractions were subjected to chromatographic analysis and each was shown to consist of a mixture of pigments, the epiphasic fraction containing the bulk of the pigment and being the more complex. The descriptions to follow are based upon a number of chromatograms of the pigment from wild type *Neurospora*.

A. COMPOSITION OF THE HYPOPHASIC PIGMENT FRACTION

No fully satisfactory procedure was found for the resolution of this fraction and, as a consequence, the number and identity of the components remain in doubt. Much of the difficulty may be attributed to the strong adsorbability of the pigment and to the large amounts of colorless contaminants. The entire fraction showed mildly acidic properties, suggesting that the components were carotenoid acids rather than xanthophylls.

This pigment formed a strongly adsorbed orange zone at the top of a $\text{Ca}(\text{OH})_2$ column which failed to migrate during prolonged washing with petroleum ether. Development proceeded slowly as washing was continued first with ether and then with ether containing 3% acetone. At this point the chromatogram presented the following appearance (the figures to the left denote the breadth of the zone, in mm.):

5	deep orange,
60	reddish orange,
5	colorless interzone,
20	brownish orange,
30	pale yellow (diffuse zone).

⁴ Separation of the hypophasic pigment from the bulk of the pigment had the added advantage of simultaneously removing large amounts of methanol-soluble impurities present in the original extract. As a result, it was possible to achieve a better resolution of the epiphasic pigments.

⁵ Not infrequently a colored precipitate appeared in the petroleum ether solution of the epiphasic pigments when the last traces of methanol were washed out. The pigments associated with this precipitate were found to be the more strongly adsorbed polyenes; i.e., those contained in section A of the chromatogram shown in Table I. To avoid quantitative losses in these pigments it was necessary to dissolve the precipitate by the addition of benzene.

Development was continued until the lower zones were washed into the filtrate and the reddish-orange zone, now diffuse, occupied the upper half of the column. The pigment from this zone could be recovered, in part, by eluting with a mixture of ether and alkaline methanol. The minor pigments of the filtrate, some of which may be isomers of the major orange pigment, were not investigated further. A characterization of the major pigment follows.

A blue color was formed when its petroleum ether solutions were treated with concentrated H_2SO_4 . Upon partition between aqueous methanol and petroleum ether the following distribution was observed: with 90% methanol, predominantly epiphase, but with a faint yellow tinge in the hypophase; with 95% methanol, major portion of the pigment in the epiphase, but with a definite yellow in the hypophase. When partitioned over alkaline methanol (5% KOH in 90% methanol), however, the pigment was completely hypophasic. Upon acidification of the methanol the partition behavior was reversed and the pigment appeared in the epiphase. It is primarily on the basis of this partition behavior that the pigment is classified as an acidic carotenoid. It was also noted that dilute, alkaline-methanol solutions were yellow but turned a yellow-orange upon acidification.

Visual spectroscopic examination of the major orange pigment revealed the presence of two broad absorption bands, the precise location of which was determined

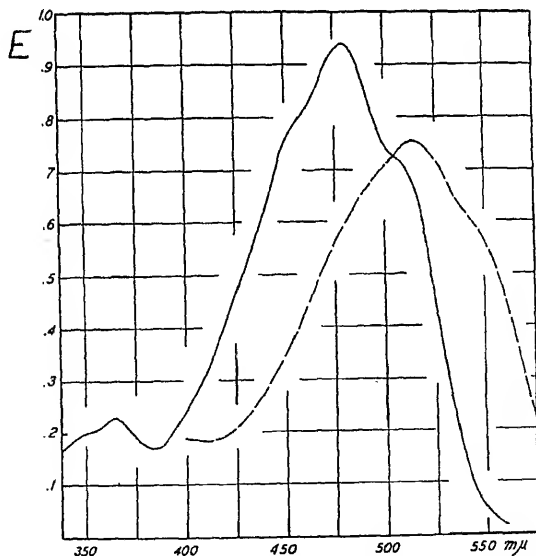


FIG. 1. Extinction curves of acidic pigment. —, in petroleum ether.
---, in carbon disulfide.

with difficulty. They were estimated at 516 and 482 $m\mu$ in petroleum ether and 558 and 504 $m\mu$ in carbon disulfide. A rapid blurring of the spectrum took place upon the addition of iodine and the bands were no longer readable. Extinction curves of this pigment are given in Fig. 1. In carbon disulfide the absorption curve is somewhat flattened and shows a single maximum at 512–514 $m\mu$. In petroleum ether the curve shows a maximum absorption at 479 $m\mu$, a strong inflection at 506 $m\mu$, and a *cis*-peak at 364 $m\mu$.

TABLE I

*Characteristic Chromatogram of the Epiphasic Pigment Mixture Obtained
From Wild Type Neurospora crassa*

(The calculated dry weight of the starting material was 75 g. The figures denote the depth of the zone in mm. on a $\text{Ca}(\text{OH})_2$ column, 58×300 mm. Development was effected with 2–3% acetone in petroleum ether. The column was cut into the four sections A–D.)

3	Orange	} A
1	Orange	
22	Rose red	
5	Pink	
5	Pale orange	} B
8	Colorless interzone	
30	Red orange	
11	Yellow orange	
4	Red	} C
20	Colorless interzone	
33	Lemon yellow	
5	Colorless interzone	
22	Orange	} D
5	Orange yellow	
10	Colorless interzone	
2	Pale orange	
10	Colorless interzone	
2	Pale orange	
Filtrate:	Orange (traces)	
	Fluorescent substance	

Acidic carotenoids are found rather frequently in company with the epiphasic pigments of *Thallophytes*, especially the *Fungi*, although for the most part little is known concerning their chemical nature or identity. One such pigment, observed by Lederer (13) in extracts of the ascomycete, *Polystigma rubrum*, showed maxima at 516, 485 $m\mu$ in petroleum ether and 550, 515 $m\mu$ in carbon disulfide. There is, however, little basis at present for identifying the acidic pigment from *Neurospora* with this or any other pigment of a similar nature described in the literature.

B. COMPOSITION OF THE EPIPHASIC PIGMENT FRACTION

Preliminary resolution of this fraction in petroleum ether was achieved by adsorption on a firmly packed $\text{Ca}(\text{OH})_2$ -Celite column. First a deep reddish-orange zone appeared at the top, and, upon further development with petroleum ether, the upper half of the column

TABLE II

Extinction Coefficients of Neurosporene and the Equilibrium Mixture of Its Stereoisomers at the Maxima and Minima

(The maxima are italicized and points of inflection indicated by an asterisk.)

In hexane			
Fresh solution		After iodine treatment	
$m\mu$	$E_{1\text{ cm.}}^{1\%}$	$m\mu$	$E_{1\text{ cm.}}^{1\%}$
<i>469</i>	3010	<i>466</i>	2130
456	1510	454	1480
<i>438.5</i>	2990	<i>436</i>	2420
425	1630	420	1570
<i>415</i>	1920	<i>413</i>	1660
*395	950		
344	115	345	174
<i>332</i>	214	<i>331</i>	413
325	180	324	312
<i>320-21</i>	188	<i>319</i>	327
290	94	290	142
267	610	<i>268</i>	517
*260	478		
In carbon disulfide			
<i>502</i>	2040		
487	1360		
<i>469</i>	2150		
450	1310		
<i>443</i>	1370		
*420	750		

became filled with overlapping zones of different colors. Near the base of the column, well in advance of the latter zones, was a narrow, pale-orange zone. In ultraviolet light it was noted that this zone was pre-

ceded by a region of strong bluish-green fluorescence. These two zones were washed into the filtrate and collected for rechromatography.

The pigment mixture remaining on the column was resolved by developing with petroleum ether containing 2-3% acetone. This resulted after 1-2 hours, in the appearance of 4 major zones and a variable number of minor zones, the number of which depended upon the degree of spontaneous isomerization. A typical chromatogram of the epiphasic pigments is given in Table I. When development was complete, the column was cut, usually into the 4 sections indicated in Table I, each section containing a single major pigment. The pigments in the lower 3 sections could usually be eluted with ethanol or acetone, although an addition of petroleum ether was sometimes required to achieve a complete recovery. The strongly adsorbed pigments of section A were best eluted with a 1:1 mixture of benzene and ethanol.

The eluate from each section was transferred to petroleum ether or benzene and each component was rechromatographed. For convenience the pigments are best considered in 5 groups, in accordance with their respective distribution in the filtrate and the 4 sections of the initial chromatogram (see Table I).

1. *Pigments of Section A.* This group of pigments was resolved from benzene on a 2:1 mixture of CaCO_3 and $\text{Ca}(\text{OH})_2$, using acetone in benzene as developer. By means of a preliminary chromatogram a separation was made into two subfractions: A-I, containing those pigments adsorbed above the major rose-red pigment; and A-II, containing the latter pigment and the zones below it.

A typical chromatogram of fraction A-II was as follows (developed with 1.5% acetone in benzene; spectral maxima are in benzene):

30	almost colorless,
10	colorless interzone,
40	dark rose-red: 549, 511, 479 $\text{m}\mu$ (with iodine, 546.5, 508, 475.5 $\text{m}\mu$),
40	pale red: 545, 506.5, 474.5 $\text{m}\mu$ (546.5, 507.5, 474.5 $\text{m}\mu$),
20	colorless interzone,
10	orange: 520.5, 486, 455.5 $\text{m}\mu$ (520.5, 485, 454.5 $\text{m}\mu$).

On the basis of evidence to follow, the major rose-red pigment was identified as spirilloxanthin, a carotenoid first isolated by van Niel and

Smith (19; *cf.* 20, 17) from the purple bacterium, *Rhodospirillum rubrum*. The pigment contained in the pale-red zone is an isomer (presumably neo A) of spirilloxanthin (20). The pigment of the bottom zone, present only in traces, showed the same spectral maxima as did the major reddish-orange pigment of section B, suggesting an incomplete separation of the pigments of groups A and B.

Absorption maxima of the rose-red pigment were as follows: in carbon disulfide, 573, 532.5, 495 $m\mu$; in benzene, 548.5, 510, 478.5 $m\mu$; in petroleum ether (b.p. 60–70°C.), 529, 493, 461.5 $m\mu$. The absorption maxima and minima were at (maxima are italicized) 546, 531, 510, 491, 480, 411, 399, 386, 380, 351, 325 $m\mu$ in benzene. These

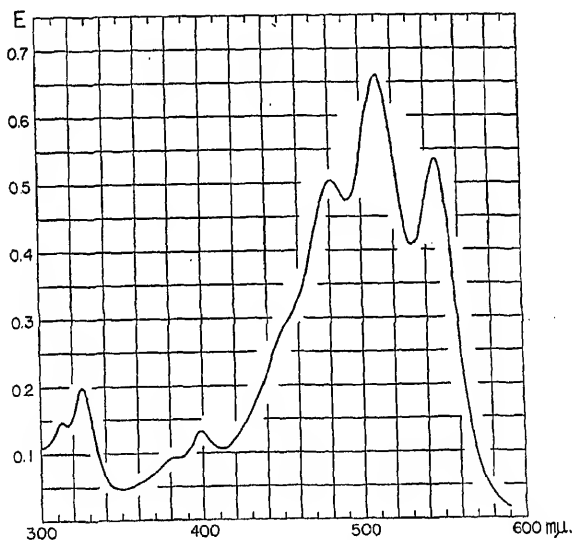


Fig. 2. Extinction curve of spirilloxanthin in benzene.

data are in excellent agreement with the published values for spirilloxanthin (20): also typical of spirilloxanthin is the extinction curve of the *Neurospora* pigment shown in Fig. 2. When fresh solutions of the *Neurospora* pigment and authentic spirilloxanthin were mixed together in equal amounts and chromatographed, the major portion of the pigment was contained in a single zone after complete development.⁵

⁵ A crystalline sample of spirilloxanthin, slightly impure, was made available by Prof. Zechmeister. A benzene solution was prepared from the crystals and chromatographed to obtain a purified sample of all-*trans*-spirilloxanthin. In solution, spirilloxanthin undergoes a rapid spontaneous isomerization (20) and, since several pigment zones appear normally, it is somewhat difficult to interpret mixed chromatograms.

Spirilloxanthin from *Neurospora* was crystallized from a small volume of CS₂ and benzene by the addition of petroleum ether. With the small quantity of pigment available, however, a chemically pure substance was not obtained. The yield of crude crystals was about 1 mg. Two crystal patterns were noted, both of which have been reported previously. Observed for the most part were clusters of petal-like crystals (19), although clusters of deep purple needles (20) were also present.

Fraction A-I, containing the minor pigments, gave the following chromatogram (development with 15-20% acetone in benzene; spectral maxima in CS₂):

13	containing an orange and a red streak,
13	deep orange: 546, 505.5, 472.5 m μ (with iodine, 542.5, 501.5, 467 m μ),
14	pale orange: 537.5, 497.5, 465 m μ (542.5, 500.5, 465 m μ),
2	purple: 539, 498.5 m μ (541.5, 499.5 m μ),
6	pale purple: 573, 534.5, 497 m μ ; spirilloxanthin,
25	diffuse lavender; neo spirilloxanthin?

The two colored streaks near the top of the column were discarded along with the two bottom zones. The spectral data indicate that the remaining three pigments are members of the same stereoisomeric set and that the pigment of the deep-orange zone is the all-*trans* isomer. Further studies of this fraction were therefore limited to the latter pigment.

The partition behavior of the orange pigment is reminiscent of certain monohydroxy carotenoids. In the partition system petroleum ether + 90% methanol a faint yellow tinge was imparted to the hypophase. When 95% methanol was used, the hypophase was a definite yellow, although the major portion of the pigment was retained by the epiphase. Crystals of the pigment were formed in small quantities upon concentration of petroleum ether solutions. These were quite soluble in benzene or carbon disulfide but only moderately soluble in petroleum ether.

The pigment displayed the following spectral maxima (visually determined): in carbon disulfide, 546, 505.5 472.5 m μ ; in benzene, 520, 485, 454 m μ ; in petroleum ether, 502, 469.5, 442 m μ . The maxima and minima in carbon disulfide, as determined with the Beckman instrument, were at 542, 526, 504, 483, 473 m μ .

Two known monohydroxy carotenoids possess properties similar to those of the orange pigment from *Neurospora*. The spectral maxima of lycopxanthin (31) in the same 3 solvents are very close to those listed

above. Also similar are the maxima of rhodopin, one of the pigments of purple bacteria (11, 12; cf. 17). Both pigments are strongly adsorbed on $\text{Ca}(\text{OH})_2$, and both are incompletely epiphasic to 95% methanol.

On the basis of the evidence at hand, a definite identification cannot be made. It is possible to state only that this minor *Neurospora* pigment is similar to lycoxanthin and rhodopin and is possibly identical with one of them. The occurrence of this type of pigment among the ascomycetes has been reported previously by Lederer (13), who observed a pigment similar to lycoxanthin in extracts of *Polystigma rubrum*.

2. *Pigments of Section B.* This fraction, in petroleum ether, was rechromatographed on $\text{Ca}(\text{OH})_2$ and developed with 7.5% acetone in the same solvent (the spectral maxima are in petroleum ether):

- 35 orange: 504, 471, 444 $\text{m}\mu$ (with iodine, 501, 468, 440 $\text{m}\mu$),
- 5 colorless interzone,
- 20 yellow orange, diffuse: 499, 465.5, 438 $\text{m}\mu$ (502, 469, 441 $\text{m}\mu$),
- 8 pale red: 506.5, 473 $\text{m}\mu$ (505.5, 472.5).

The major orange pigment was identified as lycopene; and, of the pigments contained in the two lower zones, at least the uppermost represents a stereoisomer of lycopene. A few previous cases are on record of the occurrence of lycopene among the cryptograms (cf. 32, 6).

Absorption maxima of the major orange pigment were at 546, 505.5, 473 $\text{m}\mu$ in carbon disulfide and 503.5, 471.5, 442.5 $\text{m}\mu$ in petroleum ether (cf. 15, for lycopene maxima). Maxima and minima, as determined with the Beckman instrument, were at 502, 488, 471, 454, 444, 374, 363, 316–318, 295 $\text{m}\mu$ in hexane. Molecular extinction curves of the all-*trans* pigment and the equilibrium mixture of its stereoisomers in hexane were also determined. Both curves are similar in shape to those given by Zechmeister *et al.* (34) for lycopene, and the values of the extinction coefficients (ash content of crystalline sample not known) are in good agreement.

About 1.5 mg. of lycopene was isolated in the pure state, the crystallizations being carried out from benzene and methanol. The sample consisted of very small carmine-red rod-shaped crystals and was free of colorless impurities. Two melting point determinations gave values of 174.8° and 170.8°C. The former agrees well with the published value for lycopene of 175°C. (cf. 16). Large crystals with the swallow-like tails characteristic of lycopene (29, p. 288) were obtained upon slow recrystallization from petroleum ether and ethanol in the cold. Final proof for the identification as lycopene was obtained from a mixed chromatogram with an authentic sample.

The pigment of the yellow-orange zone below lycopene is presumably *neo*-lycopene A, for which Zechmeister *et al.* (34) give the following maxima in petroleum ether: 499, 467.5, 438 $\text{m}\mu$; with iodine, 502, 471,

441.5 $m\mu$. These values are essentially the same as those listed above for the yellow-orange pigment.

It has not been possible to identify with certainty the minor red pigment of the bottom zone in the above chromatogram. Although adsorbed below lycopene, this pigment shows maxima at slightly longer wave lengths, and these decrease upon the addition of iodine. These facts suggest that the pigment is not a *cis* isomer of lycopene but a naturally occurring all-*trans* pigment. The possible existence of a pigment similar to, but not identical with, lycopene has been suggested by Karrer and Solmssen (11) in the case of rhodopurpurin, one of the

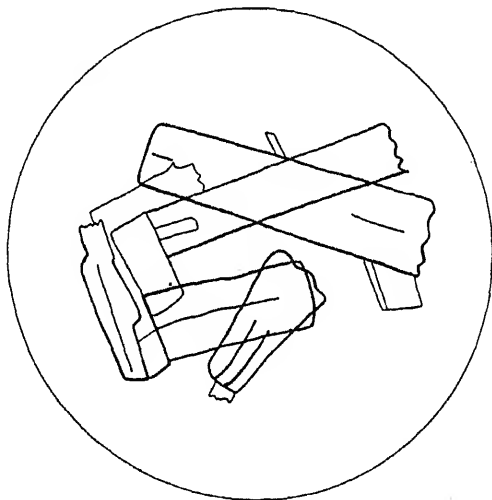


FIG. 3. Neurosporene, crystallized from benzene and methanol.

purple bacteria pigments. In petroleum ether the spectral maxima of the *Neurospora* pigment, 506.5 and 473 $m\mu$, are not very different from those of rhodopurpurin, 502 and 472 $m\mu$.

3. *Pigment of Section C.* The single lemon-yellow pigment contained in this section could not be identified as a known carotenoid. It was isolated in a pure state and named "neurosporene."

a. *The Isolation and Characterization of Neurosporene.* Solutions of the pigment obtained by elution of the adsorbate were accompanied by colorless impurities which prevented initial crystallization from ben-

zene and methanol.⁷ The pigment could be crystallized from CS₂ and ethanol, although with a low yield. Recrystallizations were then effected without difficulty from benzene and methanol.

Macroscopically the purified product was dull orange-red and showed no glitter in strong light. Microscopic examination revealed very small prismatic plates, many of which had jagged ends (Fig. 3). Thin crystals were a pale brownish-yellow, whereas thick individuals or overlapping thin ones showed a warm brown color. Colorless impurities were absent. A different pattern resulted when the pigment was recrystallized slowly, in the cold, from petroleum ether and 96% ethanol.

The individual crystals were large, somewhat diamond-shaped, and, for the most part, grouped together by 4's or 5's into symmetrical clusters. A total of 5.6 mg. purified neurosporene was obtained. The crystals melted sharply at 123.8°C. With the limited amount of material available, only a single analysis was made:

Analysis: 2.106 mg. subst.; 6.442 mg. CO₂, 1.976 mg. H₂O, 0.125 mg. ash.

		%C	%H
Calcd.:	C ₄₆ H ₅₆ ,	89.48	10.52
Calcd.:	C ₄₆ H ₅₈ ,	89.15	10.85
Calcd.:	C ₄₆ H ₆₀ ,	88.81	11.19
Found (after cor. for ash):		88.74	11.16
Mol. weight: 0.491 mg. subs. in 4.587 mg. exaltone; m.p. 62.5°C.; Δ = 3.1°.			
Calcd. mol. wt. for C ₄₆ H ₆₀ , 540; found, 510.			

The ash content of the sample was high (almost 6%). Nevertheless, definite conclusions may be drawn from this analysis because the sample was known to contain glass particles. The formula, C₄₆H₅₈ ± 2H, is tentatively assigned to neurosporene.

The pigment in petroleum ether is completely epiphasic to 90 and 95% methanol. Crystals of neurosporene are only slightly soluble in methanol, somewhat more soluble in ethanol, and quite soluble in petroleum ether, benzene, or carbon disulfide. A blue coloration was formed when petroleum ether solutions were treated with concentrated H₂SO₄. Upon treatment with 85% phosphoric acid, no color change was observed and the pigment remained in the petroleum ether phase. The position of neurosporene on Ca(OH)₂ (developed with 2-3% acetone in petroleum ether) is below lycopene and above γ-carotene. The visually observed spectral maxima of neurosporene are at shorter wavelengths than those of the well-known carotenes: in carbon disul-

⁷ Readsorption of the pigment and prolonged washing on the column failed to remove the impurities. Likewise no improvement was effected after saponification and repeated extraction of the solutions with 90% methanol.

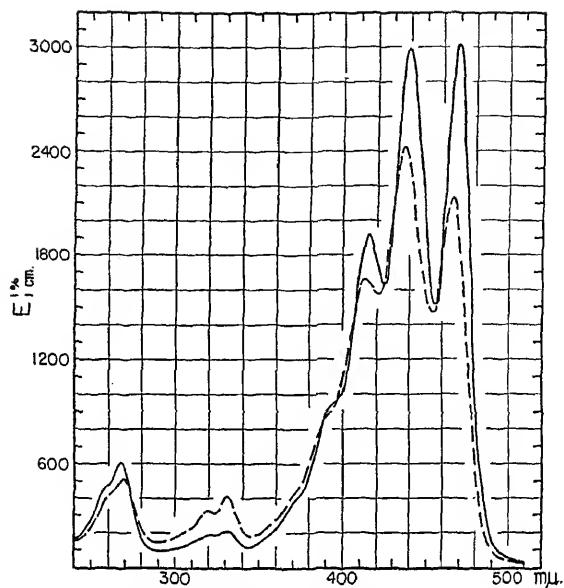


FIG. 4. Extinction curves of neurosporene in hexane. —, fresh solution of all-*trans* compound. ---, mixture of stereoisomers after iodine catalysis.

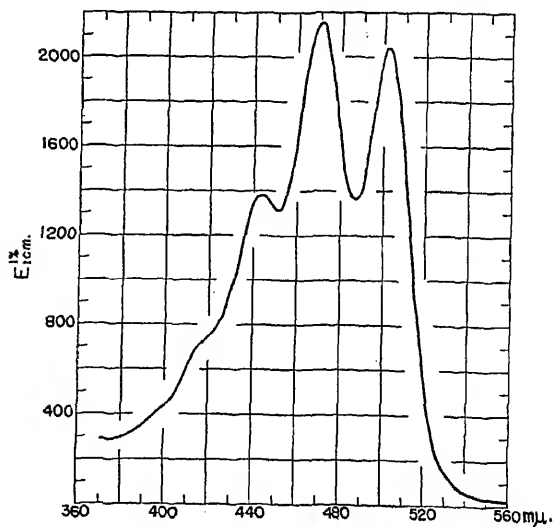


FIG. 5. Extinction curve of neurosporene in carbon disulfide.

fide, 502.5, 470.5, 439.5 $m\mu$; in petroleum ether, 470, 441.5 $m\mu$. The bands in petroleum ether are especially sharp. In this solvent three bands were apparent, one of which was located in the far violet and could not be determined accurately by visual means; upon the addition of iodine, the main maxima shifted to 468 and 438.5 $m\mu$, indicating that neurosporene occurs in the all-*trans* configuration.

In Fig. 4 are presented the extinction curves of all-*trans*-neurosporene and partly isomerized neurosporene in hexane and, in Fig. 5, all-*trans*-neurosporene in CS_2 . Upon iodine catalysis, the pigment shows a response typical of other carotenoids (*cf.* 30). Of interest in the case of the all-*trans* pigment in hexane is the presence in the visible portion of the spectrum of two absorption peaks of almost identical height. In Table II are listed the extinction coefficients at the maxima and minima of a 1% solution of neurosporene in hexane and CS_2 .⁸ For use in quantitative estimations of neurosporene, the following coefficient is recommended: $E_{1cm}^{1\%} = 2990$ at 438–439 $m\mu$ in hexane.

Although different in other ways, neurosporene shows a striking similarity to 5,6-dihydro- α -carotene (22,35) not only in the location of the spectral maxima and minima, but also in the shape of the extinction curves. The similarity also extends to the absolute values of the extinction coefficients.⁹ In the case of 5,6-dihydro- α -carotene, Polgár and Zechmeister (22) have pointed out that the location of the spectral maxima are indicative of the presence of 9 conjugated double bonds. It therefore seems to follow that the chromophoric grouping in neurosporene also contains 9 conjugated double bonds.

b. Comparison of Neurosporene with Similar Yellow Pigments. A variety of epiphasic pigments have been reported in the literature which display spectral maxima almost identical with those of neurosporene. In some cases, however, the pigments have not been adequately characterized.

⁸ The hexane solution used in these determinations was made directly from crystals. The CS_2 solution, however, was prepared from an aliquot of the stock hexane solution which had been evaporated to dryness. In view of the greater opportunity for isomerization, the values reported for the pigment in CS_2 are less reliable than those in hexane.

⁹ Zechmeister and Polgár (35) have reported the molecular extinction coefficients of 5,6-dihydro- α -carotene in hexane. To provide a basis for comparison, the coefficients listed for neurosporene were recalculated as mol. extinction coefficients, assuming a m.w. of 538.

Neurosporene is apparently identical with "pigment A" recently observed by Bonner, Sandoval, Tang and Zechmeister (2) as an unidentified minor component present in *Rhodotorula rubra*. This conclusion is based mainly upon the following considerations: homogeneity was indicated in a mixed chromatogram;¹⁰ and no appreciable differences in spectral maxima and minima (in petroleum ether) could be detected with the Beckman instrument.

Monoepoxy- α -carotene (10), diepoxy- β -carotene (10), and 5,6-dihydro- α -carotene (22,35), can be differentiated from neurosporene on the basis of their melting points. The adsorption behavior of 5,6-dihydro- α -carotene is very different from neurosporene, as was shown by a mixed chromatogram. In the system petroleum ether-calcium hydroxide neurosporene was adsorbed at the top of the column, while 5,6-dihydro- α -carotene was washed into the filtrate.

Flavorhodin, a pigment isolated from purple bacteria by Karrer and co-workers (11,12), differs from neurosporene in its melting point and adsorbability.¹¹ The latter criterion may also be applied to ϵ -carotene isolated by Strain and Manning (24) from the diatom *Navicula torquatum*.

Melting points and data on adsorption behavior relative to the common carotenes have not been reported for sarcinene¹² (3), the pigment of *Flavobacterium sulphureum* (23), and the hydrocarbon pigment from chicken retinas (25). The status of these pigments relative to neurosporene and other yellow pigments with similar chromophores is, therefore, uncertain.

4. *Pigments of Section D.* The major orange pigment and the orange-yellow pigment of this section could be separated only with difficulty. Chromatographically homogeneous solutions of these pigments displayed the following maxima in petroleum ether: orange pigment, 493, 460.5, 433 $m\mu$ (with iodine, 490, 459 $m\mu$); orange-yellow pigment, 487, 456, 429 $m\mu$ (486.5, 455.5, 429 $m\mu$). Spectral examination of the minor pale-orange pigments (see Table I) before, and after iodine catalysis, showed them to be isomers of the major orange pigment. The latter was identified as γ -carotene (for the occurrence of this pigment among cryptogams cf. 2, 5, 6, 13).

¹⁰ A small amount of "pigment A" in solution was made available by A. Sandoval.

¹¹ Flavorhodin is reported as being adsorbed on $\text{Ca}(\text{OH})_2$ below a pigment zone which appeared to contain β -carotene. On the basis of a recent study by van Niel (18), there is reason to question the identity of the latter pigment.

¹² Chargaff and Dieryck (3) reported the spectral maxima of sarcinene in petroleum ether only, the maxima being almost identical to those of neurosporene. In a recent list Karrer (9) gives the maxima of sarcinene in CS_2 at 490, 460, 433 $m\mu$, these values differing considerably from those of neurosporene.

The γ -carotene fraction from *Neurospora* was crystallized from benzene and methanol with a yield of 26 mg. The crystalline preparation contained a mixture of large and small brownish-orange rhomboids, with smaller crystals of indefinite shape. Samples recrystallized from petroleum ether and ethanol showed a different pattern (slender needles aggregated, for the most part, into rosettes).

The m.p. was not very sharp, the average of 4 determinations being 152°C. Zechmeister and Schroeder (37) report 150°C. for γ -carotene from *Mimulus*.

Analysis: 2.144 mg. subst.; 6.934 mg. CO₂, 2.075 mg. H₂O, 0.016 mg. (0.748%) ash.

Calcd. for C ₄₀ H ₅₆ :	C 89.48	H 10.52
Found:	C 88.92	H 10.91

The pigment displayed maxima at 532.5, 495.5, 460 m μ in CS₂ and 494, 461.5, 433.5 m μ in petroleum ether (b.p. 60–70°C.). The maxima and minima as determined with the spectrophotometer were at 492, 478, 461, 443, 435–436, 310–312, 282 m μ in hexane. The general shape of the extinction curves of the all-*trans* and isomerized pigments are in accord with published curves of γ -carotene (34). The determined molecular extinction coefficients, however, are about 16% lower than the published values. The reason for this discrepancy is not apparent. Identity as γ -carotene was confirmed by a mixed chromatogram.

The available evidence indicates that the minor orange-yellow pigment is δ -carotene. In CS₂ the *Neurospora* pigment displayed maxima at 525.5, 489, and 456 m μ , whereas the values given by Winterstein (27) are 526, 490, and 457 m μ .

5. *Polyenes in the Filtrate.* The orange pigment and the fluorescent component of the filtrate were resolved by chromatographing on Ca(OH)₂, although in some cases a better resolution was obtained on a 4:1 mixture of calcium hydroxide and alumina. A clean separation of the two zones was achieved by cutting the column under ultraviolet light.

The minor orange pigment was identified as β -carotene on the basis of a mixed chromatogram and the following spectral data (cf. 35, 21): in CS₂, 520.5, 485.5, 449.5 m μ ; in petroleum ether, 485, 452.5 m μ ; spectral maxima and minima, 478, 466–468, 450, 429 (inflection) m μ in hexane.

As reported previously (33), the fluorescent substance is phytofluene. The occurrence of this colorless C₄₀-polyene in *Neurospora* is of interest because of its possible role as an intermediate in the biosynthesis of carotenoid pigments (2,36).

The present status of knowledge concerning the identity of the *Neurospora* polyenes is summarized in Table III, only the naturally occurring components being listed. The identity of most of the minor pigments could not be established with certainty. Although traces of

other pigments have been observed in certain chromatograms, it is not known whether these represent normal components or merely isomerized pigments.

TABLE III

Components of the Polyene Mixture Present in Neurospora crassa

(The polyenes are listed in the order of decreasing adsorption affinity in the system calcium hydroxide-petroleum ether. Major components are italicized.)

Color of zone	Name of polyene
Red orange	Unidentified acidic pigment
Orange	Lycoxanthin or rhodopin (?)
Rose red	<i>Spirilloxanthin</i>
Red orange	<i>Lycopene</i>
Pink	Rhodopurpurin (?)
Yellow	<i>Neurosporene</i> (proposed name)
Orange	<i>γ</i> -Carotene
Orange yellow	<i>δ</i> -Carotene(?)
Orange	<i>β</i> -Carotene
Colorless	<i>Phytofluene</i>

TABLE IV

Polyene Content in Extracts of Neurospora crassa Grown in the Light and in Darkness

(The acidic pigment fraction was not determined. Minor pigments other than *β*-carotene were estimated along with a major pigment. Figures represent mg. polyene/100 g. dry wt. of mold.)

Polyene	Grown in darkness		Grown in the light	
	I	II	III	IV
Lot.....				
Spirilloxanthin	1.5	2.4	4.4	8.8
Lycopene	(2.6)	6.1	8.8	10.0
Neurosporene	5.9	6.0	5.6	5.5
<i>γ</i> -Carotene	4.3	4.3	8.0	7.2
<i>β</i> -carotene	0.16	0.13	0.4	0.25
<i>Sum of carotenoids</i>	14.5	18.9	27.2	31.8
Phytofluene	3.7	3.7	2.9	3.0

V. Spectrophotometric Determinations of the Polyenes in Neurospora

The polyenes were extracted from cultures of wild type *N. crassa* which had been incubated for 14 days at 31°C. One culture series was grown in the dark, and the other was illuminated throughout the growth period. The harvest (mycelia and conidia) from each of the two series was divided into two more or less equal lots (the calculated dry weight varied between 59.5-75 g. per lot), and each was extracted separately in

the usual manner. The growth from the dark-grown cultures was harvested and extracted in red light.¹³

In each case the hypophasic pigment fraction of the extract was discarded. The epiphasic polyenes were resolved on a column which was then cut into the 4 sections indicated in Table I, each section containing a major pigment. (Isomerized and minor pigments were recombined with a major pigment for the determinations.) β -Carotene and phytofluene were in some cases estimated together in the unresolved filtrate.

The results of duplicate estimations of the polyenes produced by *Neurospora* when grown in the light and in darkness are given in Table IV. It is clear that both phytofluene and the carotenoids are formed in the dark¹⁴ but only the production of the colored polyenes is stimulated by illumination during the growth period (*cf.* 33). Changes in the 4 pigment fractions, spirilloxanthin, lycopene, γ -carotene, and β -carotene account for the observed, almost two-fold increase of total carotenoid in the light. Apparently illumination failed to stimulate the synthesis of neurosporene under the conditions tested.

ACKNOWLEDGMENTS

The author is indebted to Dr. C. B. van Niel and Dr. A. C. Giese for their help and criticism during the preliminary studies conducted at the Hopkins Marine Station and Stanford University, and, furthermore, to Dr. G. W. Beadle for the *Neurospora* cultures and his interest in the work. The investigation was completed at the California Institute of Technology.

It is a pleasure to thank Dr. L. Zechmeister for his advice and the authentic samples, the Biology Department for providing research facilities, and Dr. G. Oppenheimer and Mr. G. Swinehart for the micro determinations.

¹³ Red light has no stimulatory effect on pigment production in *Neurospora*. van Deventer (4) has shown that the effective wave lengths are contained in a broad spectral region extending from 510 to 366 m μ . Her findings have been confirmed in a cursory way as follows. Colorless, dark-grown slant-cultures of the mutant, No. 5801, were exposed, respectively, to white light, red light (Corning filter No. 3480), and blue light (Corning filter No. 5562). A water-cooled photoflood lamp served as a light source, the cultures being placed equidistant from the source. The exposure series in each case was $\frac{1}{2}$, 1, and 5 min. After 10 hr. subsequent incubation in the dark the cultures were reexamined and the extent of the color change noted. Those exposed to blue and white light showed a graded color change of about the same magnitude for comparable exposures. Cultures irradiated with red light remained colorless. It was concluded that the effective wave lengths fell within the transmission limits of the blue filter; *i.e.*, approximately 520–380 m μ .

¹⁴ The cultures contained both mycelia and conidia. Conidia are visibly pigmented even when formed in darkness, whereas the mycelium is usually colorless (the mycelium of older cultures may also contain pigment). The color change observed upon exposure to white light is always most marked within the mycelium.

SUMMARY

The orange coloring matter of *Neurospora crassa* consists of a complex mixture of both epiphasic and hypophasic carotenoids. The four major epiphasic pigments were isolated in crystalline form. Three of these were identified as lycopene, γ -carotene, and spirilloxanthin. The fourth is a new, lemon-yellow carotenoid, $C_{40}H_{58} \pm 2H$, for which the name "neurosporene" is proposed. Of the four minor epiphasic carotenoids observed, only β -carotene was identified. One of these shows properties similar to lycoxanthin and rhodopin; the two others were tentatively identified as δ -carotene and rhodopurpurin.

The hypophasic pigment fraction, which comprises only a small portion of the total pigment, was found to contain at least one unidentified acidic carotenoid.

Pigment extracts from *Neurospora* contain a rather high concentration of the colorless, fluorescent polyene, phytofluene. Both the carotenoids and phytofluene are formed in the dark, but only the pigment production is increased in the light.

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Studies on the Microbiological Degradation of Wool.

I. Sulfur Metabolism

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INTRODUCTION

This laboratory has been interested in the biochemical mechanism of degradation of wool by fungi, particularly *Microsporium gypseum*. The present paper attempts to explain what happens when the fungus attacks the sulfur portion of the keratin molecule. While much has been accomplished in explaining the paths of sulfur metabolism in animals, as ably reviewed by Fromageot (1), no biochemical work on wool and sulfur metabolism of *M. gypseum* has been noted in the literature.

EXPERIMENTAL

A. Materials and Analytical Methods

Scoured sheep wool was defatted with alcohol and ether, rinsed in distilled water and redried at room temperature. It was then chopped by a single passage through a 40-mesh sieve in a Wiley Mill, which gave lengths of fiber averaging between 30 and 200 μ .

The fungus primarily used was *Microsporium gypseum* PQMD 196, which had been isolated from woolen fabric. Two other fungi were also used:—*Scopulariopsis brevicaulis*, Aust 69, and *Aspergillus niger*, PQMD 4j.

Four basic media were used throughout this work and are hereafter referred to by the numerals indicated below:

	I (Wool suspension)	II (Peptone- dextrose)	III (Proline- dextrose)	IV (Tyrosine- dextrose)
	g./l.	g./l.	g./l.	g./l.
MgCl \cdot 6H $_2$ O	1.68	1.68	1.68	1.83
K $_2$ HPO $_4$	2.09	2.09	2.09	2.21
KH $_2$ PO $_4$	2.68	2.68	2.68	2.50
NH $_4$ NO $_3$	—	—	—	3.00
Peptone	—	10.0	—	—
Dextrose	—	10.0	1.00	5.00
L-Proline	—	—	1.00	—
L-Tyrosine	—	—	—	5.00
Wool	50.0	—	—	—

Cystine and cysteine were determined by means of the Kassell and Brand (2) photometric method. Sulfur was determined gravimetrically as sulfate, and, in the case of solid residues, after oxidation in a bomb. Hydrogen sulfide was sought by absorption in cadmium acetate and examined quantitatively by means of the color produced by *p*-aminodimethylaniline monohydrochloride (diamine reagent). The method for determination of intermediate oxidation products was that of Lavine (3); thiosulfate by iodometric titration. The photometric method of McCarthy and Sullivan (4) was used for methionine assay.

B. Shake Flask Technic for Study of Action of Fungus on Wool

A 22-l. Pyrex round-bottom short ring-neck flask was used as the reaction chamber. A train for aeration was set up as follows: (a) air supply, compressed air; (b) large air filter capable of being sterilized in an autoclave; (c) cadmium acetate gas washer, to trap any hydrogen sulfide; (d) boric acid gas washer, to trap any ammonia vapors; (e) 10-l. Pyrex bottle containing 6 l. distilled water, to insure a moist air supply to the main reaction flask continuously throughout the experiment; (f) the main reaction flask; (g) two boric acid gas washers for the adsorption of ammonia. Cadmium acetate gas washers for the absorption of H_2S were omitted from the end of this train, since it had been shown repeatedly in preliminary experiments that no H_2S was given off as a result of the action of *M. gypseum* on wool or sulfur-containing amino acids.

The main reaction flask was filled with 5 l. of Medium I containing 5% of wool, which served as a sole source of C, N and S for the growth of the microorganisms. To insure sterile conditions before inoculation, that part of the train indicated by b, c, d, e, and f were sterilized for 1 hr. at 120°C. Prior to inoculation, the train was aerated for an hour to blow out gases arising from the partial decomposition of the wool during autoclaving (5).

Aeration was at the rate of approximately 600 ml./min. The reaction flask was mounted on a horizontal shaker which oscillated through a 4 in. path at the rate of 90 cycles/min. Samples were withdrawn aseptically while the apparatus was moving after 4, 8, 11, 14, and 21 days growth, respectively.

The insoluble residue from the aliquot taken was separated by centrifugation, washed twice with 95% ethyl alcohol and then ethyl ether and dried to constant weight in a vacuum oven at 70°C. The mycelium was determined on 5 g. portions of the dried total solids. They were placed in a flask and brought just to boiling in 10% NaOH, whereupon all the wool and about 45% of the mycelium dissolved.

C. Metabolic Studies on Sources of Sulfur Other than That of Wool

Stock cultures of the fungi were grown in flasks containing nutrient solution II (peptone-dextrose). In the case of the experiments on cystine and cysteine and their partially oxidized derivatives, the quantity of mycelium used in each determination was the total of the 4 day growth at 30°C. of three 500 ml. Erlenmeyer flasks containing 150 ml. of nutrient solution each. To measure the end-products of metabolism on a known substrate, the fully-grown mycelium was washed aseptically by centrifuging, washing twice with sterile distilled water and finally resuspending in Medium III

(proline-dextrose). Although oxidation of cystine takes place with cells suspended in 0.5 *M* phosphate buffer, pH 7.0, a medium devoid of any source of N, C, and S other than the specific substrate added, autolysis of the cells seems to occur. This was indicated by the fact that the nitrogen in the filtrate was 2-3 times that of the nitrogen of the added substrate in the 0.5 *M* phosphate buffer. When substrate plus L-proline was used, however, the nitrogen in the filtrate equalled the sum of the substrate and proline nitrogen. When dextrose and proline were added as additional nutrients, an increase in weight of mycelium was noted, and when left out, a loss of weight resulted.

An aeration train, similar to that previously described, was used with a stationary 500 ml. round-bottomed reaction flask. A known quantity of substrate (cystine, cysteine, etc.) was placed in the reaction flask, the rubber stopper and glass tubing inserted, and the whole sterilized by autoclaving for 20 min. at 15 lbs. pressure. One hundred and fifty ml. of sterile nutrient solution III (proline-dextrose) containing the washed mycelium were added aseptically to the flask and aeration allowed to proceed. Results were discarded whenever contamination was observed.

Upon completion of a run, the mycelium was separated from the medium by centrifugation and washed with distilled water. The supernatant culture medium and washings were combined and made up to volume for subsequent analyses.

When working with methionine, it was found advantageous to increase the size of the equipment and to use a larger amount of cells, to aid in the greater production of the methyl mercaptan formed. The growth of cells from 1 l. of medium II (peptone-dextrose) was used in a 2-l. Erlenmeyer flask placed in the aeration train.

When the mycelium of the fungus was incubated with L-cystine, both 250 and 100 mg. L-cystine (1.04 and 0.42 millimoles) were used, and the results with either quantity were found to be almost identical in the recovery of metabolic products. Similar results were obtained when using 250 and 100 mg. L-cysteine (2.06 and 0.82 millimoles). In the case of L-cystine disulfide¹, 100 mg. or 0.37 millimole was used, and 100 mg. or 0.67 millimole of L-cysteine sulfinic acid¹ was used. The quantities of DL-methionine used are given in Table IV.

D. Effect of Source and Concentration of Sulfur on the Growth of M. gypseum

Medium IV (tyrosine-dextrose) was used, to which the sulfur sources and quantities indicated in Table III were added. Triplicate samples were placed on a shaker and allowed to grow for 13 days at 30°C. The mycelial growth was harvested by filtering through tared sintered glass crucibles of medium porosity. They were then washed and dried approximately 16 hr. at 100°C.

RESULTS AND DISCUSSION

A. Sulfate Formation from Organic Sulfur of Wool by M. gypseum

Analyses of the wool used in these experiments, corrected for moisture and ash, showed 16.67% N, 2.69% S, and 8.00% cystine.

¹ These compounds were kindly supplied by Mr. Norman Floyd of the Lankenau Hospital Research Institute, Philadelphia.

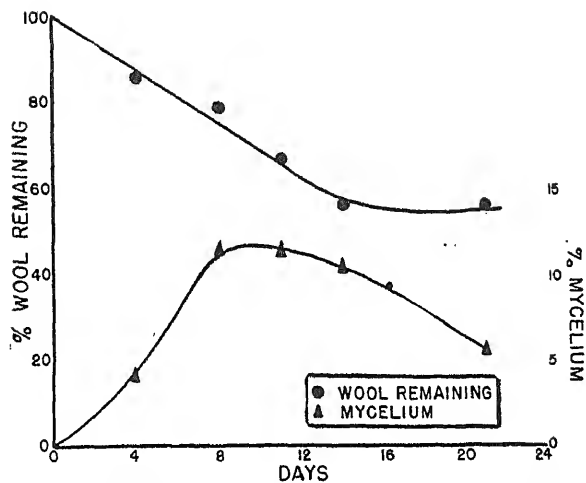


FIG. 1. Changes in wool and mycelium in shake-culture.

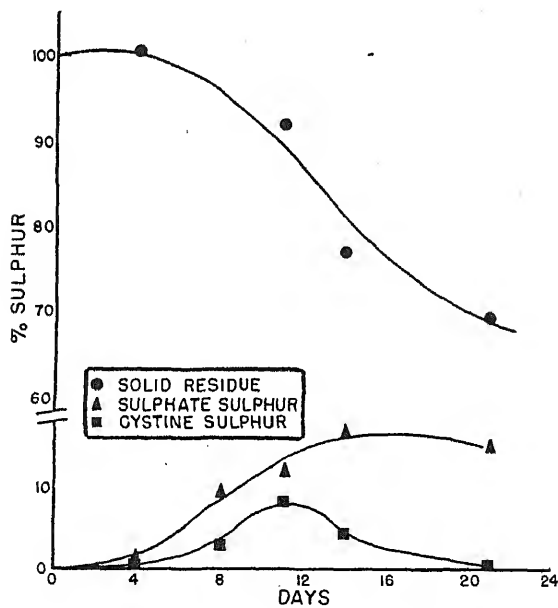
FIG. 2. Changes in the sulfur of wool degraded by *M. gypseum*.

TABLE I
*Estimation of Metabolic Products Produced by M. gypseum on
 Various Organic Sulfur Compounds*

Substrate	Time of aeration	Recovered as per cent, calc. in terms of substrate recovery						Total recovery
		Sulfate	Cystine	Cysteine	Cystine disul- foxide	Cysteine sulfinic acid	Hydrogen sulfide	
L-Cystine	hr.							
	4 c	0	96.4	0	0	0	0	96.4
	4 e	+	95.0	0	0	0	0	96.0
	18 c	0	96.0	0	—	—	—	96.0
L-Cysteine	18 e	98.2	0	0	—	—	—	98.2
	4 c	0	100.0	0	0	0	0	100.0
	4 e	6.4	82.5	0	0	0	0	88.9
	18 c	0	100.0	0	0	—	0	100.0
Cystine disulfoxide	18 e	32.6	59.2	0	+	—	0	91.8
	4 c	0	1.0	0	95.0	—	0	96.0
	4 e	15.7	32.2	0	41.5	—	0	89.4
	18 c	0	27.0	0	73.0	—	0	100.0
Cysteine sulfinic acid	18 e	77.8	2.5	0	0	—	0	80.3
	4 c	0	0	0	—	97.0	0	97.0
	4 e	3.3	0	0	—	89.8	0	93.1
	18 c	0	3.5	0	—	92.7	0	96.2
	18 e	66.0	0	0	—	0	0	66.0

— = not determined

c = control

0 = not detectable

e = experimental

+ = trace detectable

Analytical values for a known aliquot, corrected for evaporation losses, are plotted in Figs. 1 and 2. Maximum growth is found at 8 days, after which there is a gradual loss in dry weight of mycelium. The wool appears to be digested at a relatively steady rate until the 14th day, whereupon it levels off. Almost paralleling the loss of wool solids with time is the loss of S in the solids of the sample withdrawn, as indicated in Fig. 2. Actually there is an increase in the per cent of sulfur

of the residual wool, but it is not our intention to suggest what this might imply at this time. The sulfur calculated as cystine sulfur (obtained by direct quantitative cystine determination on the filtrate) rises to a maximum in 11 days and then gradually declines, almost entirely disappearing by the 21st day. Meanwhile, sulfate sulfur increases constantly, leveling off after the 15th day. The sulfate sulfur appears to stay constant at the expense of the cystine sulfur, indicating an oxidative enzyme system bringing about this conversion.

*B. Metabolism of M. gypsum on Certain Forms of Sulfur
Other Than That of Methionine*

Averages of replicate experiments on the various substrates are summarized in Tables I and II. These data bring out the following

TABLE II
*Estimation of Metabolic Products Produced by M. gypsum
on Various Sources of Sulfur*

Substrate	Time of aeration	Recovery as per cent, calc. in terms of substrate recovery					Total recovery
		Sulfate	Cystine	Cysteine	Thio-sulfate	Hydrogen sulfide	
Thiosulfate (+ 1% dextrose)	4 c	—	—	—	100.0	0	100.0
	4 e	0	0	0	98.2	0	98.2
	18 c	—	—	—	101.0	0	101.0
	18 e	0	0	0	99.8	0	99.8
Wool	7 days e	14.4*	3.5†	0	—	—	
L-Cystine (anaerobic conditions)	18 c	0	100.0	0	—	0	100.0
	18 e	0	100.0	0	—	0	100.0
Action of cell-free filtrate on cystine	18 e	0	98.0	0	—	0	98.0

— = not determined

0 = not detectable

+ = trace detectable

* = % of total sulfur in wool used

c = control

e = experimental

† = % of total cystine in wool used

facts:

1. The sulfur of cystine is only slightly converted by the fungus to sulfate at the end of 4 hr. but the oxidation is practically complete at the end of 18 hr.

2. Cysteine is completely oxidized to cystine in the absence of the fungus, simply by aeration. It is well known that traces of metals will catalyze the conversion. In the presence of the fungus a small amount of sulfate was formed, the remainder going to cystine at the end of 4 hr. However, at the end of 18 hr. approximately $\frac{1}{3}$ of the total cysteine has been oxidized to sulfate and the remainder to cystine.

3. Cystine disulfoxide, in the control, is slowly converted to cystine. However, in the presence of *M. gypseum*, sulfate appears. It has been established by Lavine (3) that aqueous solutions of cystine disulfoxide undergo dismutative decomposition, or hydrolytic oxidation and reduction, resulting in the formation of cystine and acid derivatives. The rate of decomposition increases with increasing pH. Thus, the sulfate that appeared was most likely the result of the oxidation of the cystine or the acid derivatives formed.

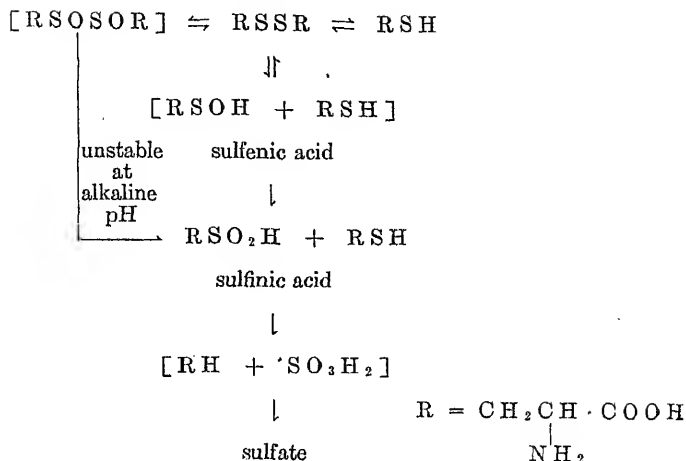
4. Cysteine sulfinic acid, as in the case of cystine, is only very slightly converted to sulfate in 4 hr., but all of the quantities accounted for converted in 18 hr.

5. Thiosulfate apparently is not further oxidized to sulfate under the conditions of the experiment. Armstrong (6) has shown that several fungi have this ability.

6. Under anaerobic conditions all the cystine was recovered as such, even after 18 hr.

The mycelium of *M. gypseum* possesses strong dehydrogenase activity as demonstrated by the Thunberg technic, using methylene blue. Cell-free filtrates from cultures grown on wool have no such activity, although they do have strong proteolytic activity. Cell-free filtrates cannot oxidize cystine in the presence of oxygen.

The data accumulated lead us to postulate tentatively the pathways of sulfur metabolism as diagramed in Fig. 3. Compounds appearing in brackets in Fig. 3 are postulated, since they have not been demonstrated in the metabolic filtrates. Cysteine is readily converted to cystine; whether this is autoxidation or enzymic catalysis cannot be stated from our data. Cystine is then hydrolyzed to cysteinesulfenic acid and cysteine. Sulfenic acid dismutates spontaneously into sulfinic

FIG. 3. Sulfur metabolism of *M. gypseum*.

acid and cysteine, according to the equation $2 \text{RSOH} \rightarrow \text{RSO}_2\text{H} + \text{RSH}$. Cysteinesulfinic acid rapidly gives rise to inorganic sulfate. When cystine disulfoxide is placed in the cycle, it too is rapidly converted to inorganic sulfate. The formation of cysteic acid and then taurine can be ruled out on the basis of practically 100% conversion of cystine to sulfate (7).

C. Inhibitory Action of Methionine on *M. gypseum*

Growth of the fungus in liquid culture containing different sources and concentrations of sulfur compounds are summarized in Table III.

TABLE III
Effect of Source and Concentration of S on Growth of *M. gypseum* (196)
mg. dry wt./culture

S source	Molar conc. of S				
	0.01	0.001	0.0001	0.00001	0
Cystine	63	70	14	13	—
Cysteine	55	67	16	11	—
Methionine	4	74	32	12	—
Na ₂ SO ₄	65	65	41	11	—
Blank	—	—	—	—	12.5

Increasing quantities of cystine, cysteine, and sodium sulfate stimulated mycelial growth. After a certain concentration level no further changes occur, regardless of further increase of the sulfur compounds. However, at somewhere above 0.001 *M* methionine there is a strong inhibition of growth.

D. Fate of Methionine in the Metabolism of Fungi

While determining the effect of *M. gypseum* on several sulfur-containing amino acids, a highly objectionable odor similar to decaying cabbage was noted when methionine was used. In contrast to the use of cystine and cysteine, only traces of sulfate were produced. Part of this volatile substance produced when methionine was used, was isolated and identified as methyl mercaptan; still some odor persisted after absorption of the mercaptan through $\text{Hg}(\text{CN})_2$. The results of the action of the various fungi in the presence of methionine as the only

TABLE IV

Estimation of Metabolic Products Formed by Several Fungi on Methionine

Microorganism	Conditions of the experiments					
	None	<i>M. gypseum</i>	None	<i>M. gypseum</i> ^a	<i>Scopulariopsis brevicaulis</i>	<i>Aspergillus niger</i>
Mg. substrate used	100	100	500	500	500	500
Millimoles used	0.67	0.67	3.36	3.36	3.36	3.36
Total liquid volume	150 ml.	150 ml.	1 l.	1 l.	1 l.	1 l.
Time of aeration	18 hr.	18 hr.	6 days	4 days	18 days	6 days
Recovered as per cent, calc. in terms of substrate recovery						
Cystine	0	0	0	0	—	—
Cysteine	0	0	0	0	—	—
Hydrogen sulfide	0	0	0	0	tr	tr
Sulfate	tr	2	tr	tr	tr	0
Ammonia	0	+	—	+	+	+
Methyl mercaptan	0	+	0	+	+	+
Methionine	98	43	98	5	—	—

tr = trace

— = not determined

+ = present, but not determined quantitatively

^a This experiment was repeated on 3 separate occasions with the same results.

sulfur source, as well as the quantitative and qualitative determination of products formed, appear in Table IV. The results with *Scopulariopsis brevicaulis* and *Aspergillus niger* agree, but *Aspergillus niger* data differ from that of Challenger (8), since this fungus brought about the formation of methyl mercaptan. It is to be noted that all experiments always showed a fission of the C—S linkage within 3 days, if at all. In the case of *M. gypseum*, 48 hr. after the start of formation of mercury methyl mercaptide crystals in the trap, no further production apparently ensued.

In those experiments where a total volume of 150 ml. of nutrient solution was used, the small amount of $\text{Hg}(\text{SCH}_3)_2$ formed appeared as crystals on the inside of the tube projecting into the $\text{Hg}(\text{CN})_2$ gas absorber. The procedure was to carefully wash the crystals down into a small beaker, centrifuge off the supernatant and rewash with distilled water. They were then taken up in a very small amount of hot anhydrous alcohol and allowed to crystallize. They were then recrystallized 3 times in this manner. When using a total volume of 1 l. of nutrient solution and 500 mg. DL-methionine in the experiments, relatively large amounts were recovered. The crystals appeared as large thin shining plates, turning greenish-brown at 160°C . and melting with decomposition at 174°C .; they did not depress the melting point of an authentic specimen, m. pt. 175°C . Found: S, 21.40%, 21.47%; $\text{Hg}(\text{SCH}_3)_2$ requires 21.75%.

These findings were of interest for several reasons. It has been shown by Birkinshaw, Findlay and Webb (9) that the fungus *Schizophyllum commune* had the ability to form methyl mercaptan (alkyl sulfide) from inorganic sulfate. The keratinolytic fungus, *M. gypseum*, with which we have been working, does not effect this synthesis when grown on inorganic sulfate or thiosulfate. Steinberg (10), working with *Aspergillus niger* and with inorganic sulfate and its products of reduction, has shown that sodium sulfoxylate (Na_2SO_2) is the lowest state of oxidation in which inorganic sulfur can be utilized efficiently, and that sulfide and disulfide were not utilized. Further, assimilability of organic sulfur varied with molecular configuration and was correlated with the presence of attached or adjacent oxygen in the molecule. Cystine, homocystine and methionine were readily available as sole sources of sulfur supply.

Challenger (8) has noted the ability of several fungi to cause fission of the —S—S— link of organic disulfides, R—S—S—R , giving the thiol R—SH and the alkyl methyl sulfide R—SMe . He also took many other organic sulfur sources and was able to show the C—SMe linkage fission. These last examples, involving methylation of organically linked sulfur, are in a somewhat different category from the direct methylation of

inorganic sulfur, as first shown by Birkinshaw *et al.* (9). This fission of the alkyl $-S-C$ link is a type of microbiological action which presents analogy with the fission of cystathionine in animal tissues.

SUMMARY

1. Degradation of wool by the action of the fungus *Microsporum gypseum* was followed analytically with respect to sulfur, using the shake-culture technic.

2. With time, there was a decrease in the sulfur in the solid-residue aliquot with an increase in the sulfate sulfur, which levels off after 13 days and stays constant. The cystine sulfur in the filtrate rose to a maximum along with the sulfate sulfur until the 11th day, whereupon it decreased, so that it was practically absent at the 21st day.

3. Inorganic sulfate is an end-product of the decomposition of wool by fungi.

4. A scheme for the metabolism of cystine and cysteine has been suggested, wherein any cysteine is converted to cystine, which, in turn, is enzymically hydrolyzed to cysteinesulfenic acid, the latter being oxidized to cysteinesulfinic acid, and finally to the sulfate ion.

5. *M. gypseum* has the ability to cause fission of the C-S linkage in methionine with the production of methyl mercaptan.

6. Methionine, as contrasted with cystine, cysteine, and sodium sulfate, caused a very definite inhibition of growth of the fungus at high concentrations. This was ascribed to the formation of toxic methyl mercaptan.

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Studies on the Biochemistry of *Tetrahymena*. XI. Components of Factor II of Known Chemical Nature¹

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INTRODUCTION

Lwoff (1) was the first to obtain growth of the ciliated protozoan, *Tetrahymena geleii* (*Glaucoma pyriformis*) in the absence of bacteria. Successful cultures were established in peptone media but all attempts to formulate a chemically defined medium failed. This was attributed to deficiencies in specific growth factors and later it was suggested that this organism had a polypeptide requirement (2). In 1941, Dewey (3) found that *Tetrahymena* requires at least 2 unidentified growth factors, which were later (4) designated Factors I and II. These factors were separable one from the other by treatment of crude materials (water extracts of egg yolk, yeast autolyzate, and timothy hay) with lead acetate. Following this treatment the Factor I activity was found in the precipitate while Factor II was not precipitated. Later (5), a third factor (Factor III) was described. In a series of investigations (6-11) the chemical natures of Factors I and III were elucidated, as well as the specific amino acid and known vitamin requirements for this organism. Factor II has been under investigation for some time in our laboratory but attempts at concentration have not been successful. Recently, concerted efforts by Dr. E. L. R. Stokstad, C. E. Hoffman and their associates at the Lederle research laboratories have yielded valuable results in the preparation of a highly active concentrate. They found that Factor II could be separated into 2 active fractions (Factors IIA and IIB) by treatment with silver nitrate. Factor IIA was precipitated by this method while Factor IIB remained in the filtrate. Factor IIA has been purified to a considerable extent and a preliminary report has been

¹ Aided by a grant from the U. S. Public Health Service and a grant recommended by the Committee on Growth acting for the American Cancer Society.

made (12) in which the name protogen has been assigned to this factor. We have been studying Factor IIB and the results of these investigations form the basis of this report. Factor IIB has been found to be a complex of known substances and, as a result, we have been able to formulate more precisely an adequate medium for the growth of this organism. We wish to express our appreciation to Dr. Stokstad and his associates for generously supplying us with protogen concentrates at various stages of its preparation, without which the major part of this work would have been impossible.

EXPERIMENTAL

Tetrahymena geleii strain W was used in these investigations. This is the strain which has been under investigation for a number of years in this laboratory. The techniques employed were the same as those recently reported (13), in that the organisms were grown in 4 ml. of medium at initial pH 7.2 before sterilization, in 15×125 mm. Pyrex tubes in a slanted position (slanting increases growth rate and yield). Incubation was for 72 hr. (unless otherwise noted) at 25°C., and all results are based on third serial transplants. Quantitative determinations of growth responses were made turbidimetrically (13). All experiments were made in triplicate and repeated varying numbers of times. At the start of this series of investigations, when crude extracts were used to supply the Factor II activity, a base medium was used similar to that previously employed (14). The amino acid concentrations were formulated on the basis of levels which were previously determined (14, Table II). The levels of

TABLE I
Dose Response with Pyridoxine

Pyridoxine HCl (γ /ml.)	Optical density after 72 hr. incubation
0	0.000
0.01	0.000
0.10	0.164
0.25	0.296
0.50	0.403
1.00	0.420
2.00	0.420
4.00	0.419

arginine, histidine, isoleucine, leucine, methionine, and phenylalanine were doubled for the present medium. Serine and threonine were increased to more than double (80 γ /ml. and 50 γ /ml., respectively) the amount given previously because of their detoxifying effects. Later determinations of the optimum levels of lysine and tryptophan gave slightly different values which were then used (40 γ /ml. and 16 γ /ml., respectively). Since valine can be synthesized to some extent, and the DL form is somewhat inhibitory, its amount was increased only to 10 γ /ml. As the work pro-

gressed, changes in the base medium were made and these changes will be discussed in turn.

TABLE II
Concentration of Amino Acids in Simulated Casein Hydrolyzate (SCH)

Essential ^a	γ/ml.
L-Arginine HCl.....	43
L-Histidine HCl.....	21
DL-Isoleucine.....	63
L-Leucine.....	97
L-Lysine.....	76
DL-Methionine.....	34
L-Phenylalanine.....	50
DL-Threonine.....	88
L-Tryptophan.....	12
DL-Valine.....	66
DL-Serine.....	77
Nonessential	
L-Glutamic acid.....	233
L-Aspartic acid.....	61
Glycine.....	5
DL-Alanine.....	55
L-Proline.....	75
L-Hydroxyproline.....	75
L-Tyrosine.....	67
L-Cysteine.....	3.5

^a The amino acids classed as essential for *Tetrahymena* are those which this organism fails to synthesize or synthesizes at a rate too low to meet its metabolic needs.

RESULTS

The substitution of protogen (Factor IIA) in the base medium for the Factor II preparations did not result in growth. This indicates that the base medium must be supplemented with something more than protogen and, accordingly, attempts were made to concentrate Factor IIB.

Early attempts at concentration of Factor IIB were carried out by various chemical procedures. Liver fraction L was treated with normal and basic lead acetate and the precipitate discarded. The filtrate was treated with silver nitrate and the resulting precipitate also discarded. The silver nitrate filtrate was high in Factor IIB activity and practically devoid of protogen so that both were required for growth. This filtrate was then treated with phosphotungstic acid in a manner similar to that described earlier (2). It was found that both the precipitate (Factor IIB') and the filtrate (Factor IIB'') were necessary for continued growth. After extended substitutions of known compounds, it was found that the activity in the phosphotungstic acid filtrate (Factor IIB'') could be replaced with Cu^{++} and Fe^{+++} ions in relatively high concen-

trations. One of the limiting factors in the growth of this organism, therefore, is a high copper requirement. No copper salts had been included in the medium up to this time as it had been felt that the slight contamination of the other salts, used in rather large amounts, would supply a sufficient quantity, if indeed copper was required. The quantity of iron which had been added as ferric chloride (1.25 γ /ml.) was found to be suboptimal and part of the filtrate activity can be traced to its iron content. At this point growth could be obtained by the use of the phosphotungstic precipitate (Factor IIB'), CuCl_2 , higher amounts of iron salts, and protogen (replacing the original Factor II).

Attempts at substitution of known compounds for Factor IIB' were made. As a result of these tests, it was found that a large portion of the activity could be replaced by increased pyridoxine. One of the constituents of the base medium was pyridoxine at a concentration of 0.1 γ /ml. This amount had been included in the earlier work and at that time it was thought sufficient, especially in view of the results, which seemed to show that pyridoxine was not an absolute requirement for this organism (10). It has become clear, however, that pyridoxine is an absolute requirement, and that from 1.0 to 2.0 γ /ml. should be added (Table I). Pyridoxal and pyridoxamine, which will be the subject of a later report, are many times more active than pyridoxine.

At this stage it could be stated that Factor II may be replaced by protogen (Factor IIA), copper and iron (Factor IIB''), and pyridoxine (Factor IIB'). Growth was far from optimal, however, and further attempts at improvement of the medium were made. The following compounds were tested for stimulatory activity: hemin; cholesterol; lecithin; oleic acid; linoleic acid; linolenic acid; arachidonic acid; vaccenic acid; Tween 20; Tween 40; Tween 60; Tween 80; Tween 85; Tween G-2144;² glutathione; streptogenin; nonessential amino acids. An extensive investigation of the levels of the essential amino acids and energy sources was also made. As a result of these tests it was found that optimum growth could be obtained by changes in the levels of the essential amino acids; the inclusion of small amounts of the nonessential amino acids, increased dextrose and acetate as energy sources, and the inclusion of Tween 85. These additions and changes will be discussed separately below.

Amino Acids

As might be expected, the interactions of the various free amino acids has proved to be the most complicated factor in the present study. In the early phases of this work, a streptogenin preparation (tryptic digest of casein (15)) was tested and found to be highly stimulatory. This stimulation was not due to streptogenin activity, however, for an amino acid mixture patterned after casein produced the same effect, at equivalent concentrations. For all routine tests, therefore, this simulated casein hydrolyzate (SCH, Table I) was added to the base medium, which had been fortified with 2.0 γ /ml. of pyridoxine, 25 γ /ml. of ferrous ammonium sulfate, and 5 γ /ml. of copper chloride.

² The various Tweens, which are polyethylene derivatives of sorbitan esterified with fatty acids (20 = lauric; 40 = palmitic; 60 = stearic; 80, 85, G-2144 = oleic), were obtained through the courtesy of the Atlas Powder Company, Wilmington, Del.

After 72 hr. incubation, the maximum growth obtained with this medium gave optical density readings of 0.276. This growth was increased to approximately O. D. 0.350 by the addition of Tween 85, the details of which appear below. To further test the possibility of strepogenin stimulation, a tryptic digest of insulin was employed. These experiments were set up to compare the activity of various concentrations of the insulin strepogenin to simulated insulin hydrolyzate (constructed from amino acids) at the same concentrations. Readings were taken on third transplant tubes at 24-, 48-, 72-hr. intervals in order to detect any possible stimulation during the early periods of growth. As can be seen from Table III, strepogenin exerts no stimulatory effect during

TABLE III
*Comparison of Insulin Digest (for Strepogenin Activity)
with Simulated Insulin Hydrolyzate^a*

Base medium is that given in Table I with the addition of pyridoxine (2 γ /ml.), protogen (0.375 γ /ml.), CuCl_2 (5 γ /ml.), $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ (25 γ /ml.), Tween 85 (500 γ /ml.), sodium acetate (1000 γ /ml.) and SCH (1X). Results expressed as optical density.

Total amount added (γ /ml.)	Insulin digest			Simulated insulin		
	Hours of incubation			Hours of incubation		
	24	48	72	24	48	72
0	.025	.216	.326	.032	.216	.326
20	.030	.197	.344	.036	.226	.335
60	.040	.167	.347	.039	.251	.348
80	.024	.177	.341	.038	.238	.343
100	.029	.189	.351	.038	.246	.347
120	.032	.203	.351	.038	.239	.356
140	.035	.198	.346	.048	.274	.354
160	.025	.170	.352	.048	.262	.355
180	.037	.178	.359	.041	.240	.339
200	.031	.142	.339	.028	.214	.351

^a The amino acid concentrations were taken from the data presented by Hawk *et al.* (16), p. 109.

any of the observed periods of growth. The slightly depressed growth with strepogenin at 48 hr. is of doubtful significance.

To determine the level of greatest stimulation by amino acids, simulated casein hydrolyzate (SCH) was added in graded amounts to the basal medium (in which the pyridoxine level had been raised, copper chloride, ferrous ammonium sulfate, protogen and 500 γ /ml. of Tween 85 had been supplied). The results are given in Fig. 1. This stimulation was further tested by a similar set of experiments in which only the non-essential amino acids of the SCH were added. These results are given in Fig. 2. From a

comparison of these 2 sets of data, it appears obvious that the most stimulation comes from the increases of the essential amino acids brought in with the SCH or by increasing the essential amino acids of the basal medium, although some stimulation can be traced to the nonessentials (Fig. 2). Simply increasing the essential amino acids alone by as much as a factor of 4 increased growth, but never to maximum concentra-

TABLE IV
Complete Medium

(All amounts are given in γ /ml. of final medium)

Amino acids		Growth factors	
*L-Arginine HCl.....	163	*Ca pantothenate.....	0.10
*L-Histidine HCl.....	66	*Nicotinamide.....	0.10
*DL-Isoleucine.....	213	*Pyridoxine HCl.....	2.00
*L-Leucine.....	247	*Riboflavin.....	0.10
*L-Lysine HCl.....	196	*Pteroylglutamic acid.....	0.01
*DL-Methionine.....	214	Biotin (free acid).....	0.0005
*L-Phenylalanine.....	110	Thiamine HCl.....	1.0
*DL-Threonine.....	238	Choline Cl.....	1.0
*L-Tryptophan.....	60	*Protogen ^a	0.375
*DL-Valine.....	96		(0.75 units)
*DL-Serine.....	317	*Yeast nucleic acid	
L-Glutamic acid.....	233	(hydrolyzed).....	100
L-Aspartic acid.....	61		
Glycine.....	5	Salts	
DL-Alanine.....	55	*MgSO ₄ ·7H ₂ O.....	100
L-Proline.....	175	*K ₂ HPO ₄	100
L-Hydroxyproline.....	75	*CaCl ₂ ·2H ₂ O.....	50
L-Tyrosine.....	67	*Fe(NH ₄) ₂ (SO ₄) ₂ ·6H ₂ O.....	25
L-Cysteine.....	3.5	*CuCl ₂ ·2H ₂ O.....	5
		FeCl ₃ ·6H ₂ O.....	1.25
Carbon source		MnCl ₂ ·4H ₂ O.....	0.05
Dextrose.....	1000	ZnCl ₂	0.05
Sodium acetate.....	1000	Tween 85.....	500

* The omission of any of the substances marked with the asterisk either greatly reduces growth or makes growth impossible. In preliminary tests we have determined the essentiality of magnesium, potassium, phosphate, and calcium ions. Ferric and ferrous iron is effective. Nucleic acid derivatives can be substituted for the hydrolyzed yeast nucleic acid.

^a Supplied as a concentrate with a potency of 2000 units/mg. of dry weight.

tions, so it appeared that the proportions of the essential amino acids found in casein were more favorable than those of the basal medium. To test this, varying amounts of the essential amino acids in the proportions contained in casein were added to the basal medium (fortified with pyridoxine, copper, iron, Tween 85, and protogen, and 0.4 times the concentration of only the nonessential amino acids found in SCH, in line with the data presented in Fig. 2). From these data the concentrations of the amino acids used in the final medium were derived (Table IV).

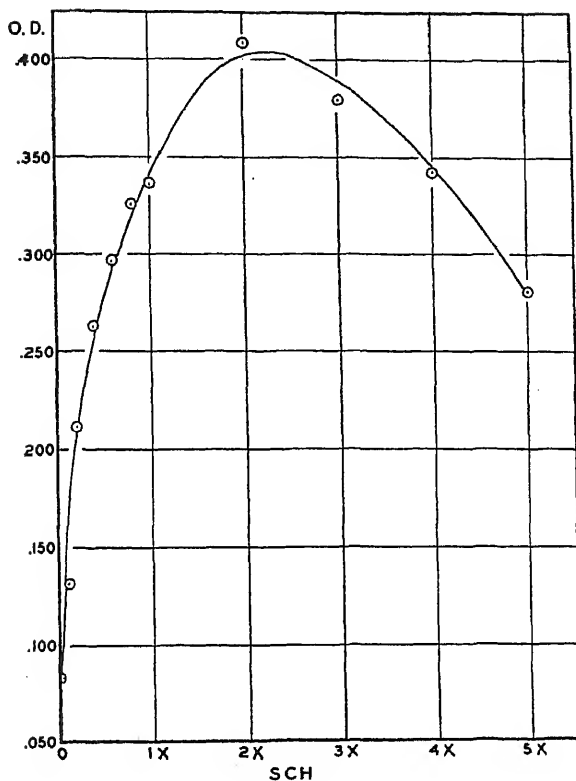


FIG. 1. Dose response to various levels of simulated casein hydrolyzate (SCH). The concentrations of the various amino acids in SCH (1X) is given in Table II. The base medium has been fortified with pyridoxine (2 γ /ml.), $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ (25 γ /ml.), CuCl_2 (5 γ /ml.), sodium acetate (1000 γ /ml.), and Tween 85 (500 γ /ml.). Growth is expressed as optical density after 72 hr. incubation at 25°C.

Tween

The question of the activity of the various Tweens and their relation to fatty acid metabolism was tested. Greatest response was obtained with the trioleate Tween 85 (Table IV), and this has been used routinely for later work. The function of the Tween may be due to its surface tension activity rather than its oleic acid content, because it could not be duplicated with any combination of the unsaturated fatty acids (oleic, linoleic, linolenic, arachidonic, vaccenic) over wide ranges of concentrations. The effect of Tween 85 could be duplicated with Tween G-2144 (also an oleic Tween) but maximum growth was reached only after a longer period of time. None of the other Tweens (20, 40, 60, 80) has a similar effect. Lecithin can replace Tween 85, but,

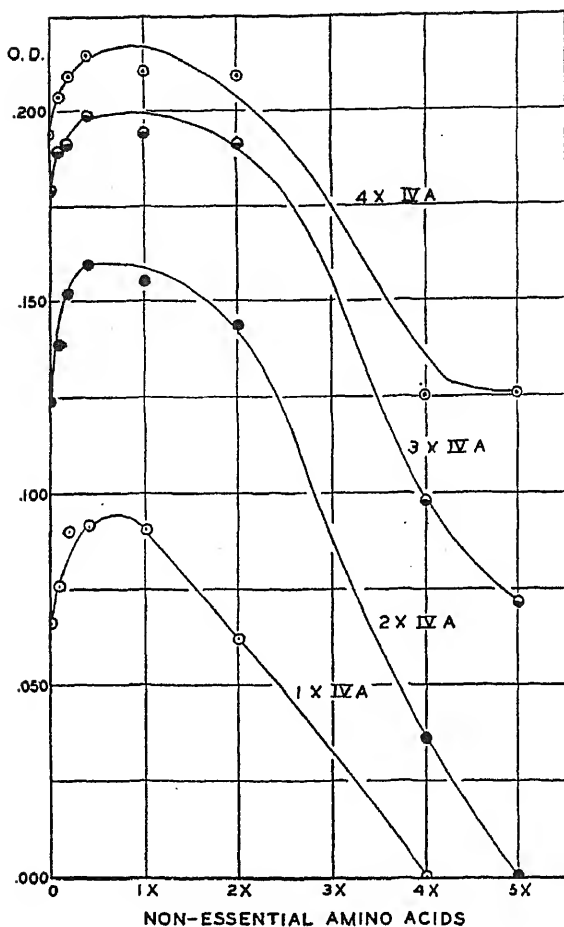


FIG. 2. Dose response to various levels of the nonessential amino acids of simulated casein hydrolyzate (SCH). IVA refers to the concentrations of the amino acids of the basal medium (1X). Growth is expressed as optical density after 72 hr. incubation at 25°C.

due to the fact that it must be dissolved in benzene or some other fat solvent in order to be added to the tubes, it was not used routinely. Tween 85 not only raises the maximum yield but increases the growth rate as well.

In spite of the fact that oleic acid exerted no stimulatory effect in low concentrations, and proved progressively inhibitory as the concentration was increased, the effect of Tween 85 may still be due to its oleic content in combination. Williams and

Snell (17) have demonstrated the detoxifying effects of the various Tweens on oleic acid. In our experience, however, the addition of oleic acid together with the Tweens (40, 60, 80) proved ineffective as a growth stimulant. In any case, it is apparent that *T. geleii* does not require long chain fatty acids as indefinitely transplantable growth occurs in their absence from the medium.

Energy Source

Dose response experiments were conducted to determine the effect of added dextrose and sodium acetate. From these data it was determined that maximum stimulation occurs with the addition of 2500 γ /ml. of dextrose, and 1000 γ /ml. of sodium acetate.

When increases in the amino acid levels were made, increasing the dextrose to as high as 4000 γ /ml., under some conditions, increased the yield. Interesting, though not unexpected, quantitative relationships were found in this connection. High levels of dextrose depressed growth in inverse relation to the amino acid concentration of the medium. High levels of dextrose were tolerated only when the amino acid levels were high. This points to the fact that the readily available source of energy in dextrose can be used only when adequate nitrogen sources for protein synthesis are present and, moreover, this energy source is deleterious when conditions allowing protein synthesis are unfavorable.

There are indications that some interference occurs when both dextrose and Tween 85 are included at their "optimum" amounts (2500 γ /ml. and 700 γ /ml. respectively). This is being investigated further and, in the meantime, the dextrose level is being held at 1000 γ /ml. and the Tween 85 level at 500 γ /ml., for routine work.

Final Medium

The composite of the results discussed above allows us to formulate a base medium for *Tetrahymena geleii* which will support excellent growth. This medium is given in Table IV.

In the light of our previous findings (6-11) several apparent discrepancies should be mentioned here. The stimulatory effects of the nonessential amino acids were not observed when crude materials were used to supply Factor II activity, but become important in the purified medium. This means that the crude materials contained traces of amino acids, even though they appeared to be amino acid-free by the ninhydrin test.

The absolute requirement for pyridoxine, riboflavin, pantothenic acid, and nicotinic acid (Table IV), in contrast to our earlier report (10), indicates that, in spite of the clear results of the *L. casei* assays used on the Factor II preparations, these assay results were unreliable. The details of the B vitamin requirements will be published separately.

The addition of the filtrate from ammonium sulfate-treated liver fraction (Factor IIB), which was used by Stokstad *et al.* (12), to our complete medium failed to increase growth rate or final yield. The same can be said for liver press cake (12), anti-pernicious anemia concentrates, and animal protein factor concentrates.³

³ These preparations were supplied by the Lederle Research Laboratories through the courtesy of Dr. E. L. R. Stokstad.

The medium given in Table IV is complete for 8 other strains of *Tetrahymena geleii* and 2 strains of *T. vorax*.⁴ It should be pointed out again that the results presented by Kline (19) are entirely incompatible with these findings. The organism he used was called "*Colpidium striatum*," which would correspond to our *T. geleii* E (18). His medium contained no stated sources of protogen, nucleic acid nor the essential amino acids histidine, leucine, lysine, and threonine.

DISCUSSION

The results of this investigation indicate clearly that the basic nutritional requirements of *Tetrahymena geleii* can be chemically defined, with the single exception of protogen. It should be pointed out that one precaution should be carefully observed in working with this medium: it must not be exposed to light for prolonged periods. Pteroylglutamic acid, pyridoxine, and riboflavin become inactivated, and growth is drastically reduced under these conditions. We have found it satisfactory to keep amino acid mixtures under toluene at low temperatures for many weeks, but the vitamin solutions should be kept separately in dark bottles, and mixtures should not be used after more than one week from the time of their combination. Tubes should always be incubated in the dark and exposed to the light of the laboratory for the minimum length of time necessary for manipulation. These precautions are not necessary when one is dealing with colored media, such as peptone or those containing crude extracts.

With the discovery and concentration of protogen (12), and the formulation of an otherwise chemically defined medium, it will now be possible to investigate in a more precise manner many problems of basic biochemical metabolism with an animal microorganism.

SUMMARY

1. The separation of Factor II, necessary for the growth of *Tetrahymena geleii*, into two fractions (IIA and IIB) by Stokstad *et al.* (12) and the concentration of Factor IIA (protogen) has made it possible to investigate the nature of Factor IIB.

2. Factor IIB can be separated into two fractions (IIB' and IIB''). Factor IIB' can be replaced by high levels of pyridoxine. Factor IIB''

⁴ *T. geleii* strains H, TP, T, GHH, E, (18); GC (the strain isolated by Glaser and Coria (19) under the name "*Trichoda pura*"); GL (the "*Glaucoma piriformis*" of Lwoff (1)); GP (the "*Glaucoma piriformis*" used by Hall and Cosgrove (20)). *T. vorax* strains V and PP (18).

can be replaced by copper salts and iron salts, in relatively high concentrations.

3. Nonessential amino acids are stimulatory and increased levels of the essential amino acids are necessary for optimum growth.

4. Tween 85 was found to be stimulatory. The evidence favors the view that the Tween exerts its stimulatory effect because of its physical properties.

5. Dextrose and sodium acetate were found to increase the yield.

6. A complete medium for *Tetrahymena* is given. With the single exception of protogen, the chemical nature of the nutritional requirements of *Tetrahymena geleii* (9 strains) and *T. vorax* (2 strains) is now known.

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Fibrinolysin and Antifibrinolysin: Biochemical Concentration of Antifibrinolysin ¹

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INTRODUCTION

Hildebrandt (1), Hahn (2), and Camus and Gley (3), were the first to report that normal blood serum has the power of inhibiting the action of trypsin. Landsteiner (4) showed that this antienzymic action was not contained in the precipitate when serum was half-saturated with ammonium sulfate, but that it was present in the albumin precipitated by complete saturation with ammonium sulfate. Opie and Barker (5) were the first to postulate that this antienzyme in serum held in check the activity of serum protease (fibrinolysin). The inhibitor compound these men worked with is presumably the compound we call antifibrinolysin which we are pleased to report has recently been concentrated and considerably purified.

Schmitz (6) published a method for preparing small quantities of a trypsin inhibitor substance from beef blood. His method and product resemble Northrop and Kunitz' (7) pancreatic trypsin inhibitor. The severity of Schmitz' method is undoubtedly responsible for the small yield he obtained, and it remains to be shown whether there is any similarity between his product from whole blood and our plasma fraction. Grob (8,9,10) has presented a series of papers giving a complete historical review of the physiological significance of the antiproteolytic activity of serum.

UNIT AND ASSAY OF ANTIFIBRINOLYSIN

Recently, we defined a unit of fibrinolysin and established an assay procedure for determining the potency of preparations by measuring their lytic action on a standard fibrin clot (11). We have subsequently

¹ This publication was presented in part at the Antienzyme Section Meeting of the American Society of Biological Chemists, May 18-22, 1947, and in part at the International Society of Hematology meeting in Buffalo, N. Y., August 23-26, 1948.

defined our unit of antifibrinolysin in terms of the fibrinolysin unit and have modified the assay procedure to establish the potency of antifibrinolysin. One unit of antifibrinolysin is defined as that quantity which will exactly neutralize one unit of fibrinolysin (11,12), buffered with imidazole at pH 7.2, in 1 hr. at 26°C.

In practice, an excess of fibrinolysin is dissolved in 0.9 ml. buffered physiological saline and 0.1 ml. antifibrinolysin solution is added. At the end of 1 hr. the excess fibrinolysin is determined as previously described, and the antifibrinolysin activity determined by difference. It has been found that 1 hr. is required for the *in vitro* neutralization of fibrinolysin and antifibrinolysin. If the time is extended another 30 min., less than 10% additional antifibrinolysin is apparent. This slower reaction time is in sharp contrast to the immediate neutralization of injected fibrinolysin by antifibrinolysin *in vivo*. Table I shows the disappearance of fibrinolysin from solution with

TABLE I
Destruction of Fibrinolysin by Antifibrinolysin

Antifibrinolysin preparation	Units fibrinolysin added	Units fibrinolysin remaining		
		30 min.	60 min.	90 min.
422	7.13	2.20	0.44	0.42
403	8.30	7.50	7.50	7.39
403	7.33	3.73	3.20	3.17
409	7.78	3.73	3.30	3.28
410	10.80	5.00	3.30	3.22
412	8.04	7.50	6.50	6.48
415	9.47	1.45	0.42	0.38

a fixed quantity of various antifibrinolysin preparations. In each case a sufficient quantity of fibrinolysin is added so that when the excess is determined the lysis time ranges between 90 sec. and 5 min. It is felt that this short exposure to the relatively high temperature of the fibrinolysin assay eliminates objection (10) to the 45°C. temperature used in the lytic assay.

A further refinement has been introduced into the assay procedure (13). After the fibrin clot is formed by the addition of the 0.6% fibrinogen solution to the thrombin-fibrinolysin mixture, a capillary tube is inserted into the clot. As soon as the clot has liquified, through the action of fibrinolysin on the fibrin, capillary attraction will raise fluid inside the capillary tube above the level of the solution in the test tube thus affording a very sharp endpoint, reproducible to within 2 sec or less in multiple determinations. This refinement eliminates removal of the test tube from the water bath and tilting to read the endpoint. An ordinary melting point tube is very satisfactory for this use.

BIOCHEMICAL CONCENTRATION

Antifibrinolysin has been prepared from bovine, equine and human plasmas, and sera by the method here presented. Slightly better results have been obtained from oxalated, rather than citrated, plasmas but it is not considered to be a critical difference. When sera were used, they were prepared from oxalated plasmas as previously described (11). Temperature control below 26°C. is not an absolute necessity but it is felt that solubility factors are reduced with lower temperatures. Hydrogen ion adjustment on the acidic precipitation is critical and affords only a narrow range between pH 3.5 and 4.0.

TYPE PREPARATION

One liter of bovine serum is cooled to +5°C. and brought to 0.50 saturation by the addition of an equal volume of saturated $(\text{NH}_4)_2\text{SO}_4$. The precipitate is removed by centrifugation in the cold. The supernatant treated serum (1 in Table II) is cooled to

TABLE II
Relative Purification of Bovine Antifibrinolysin

	Total units	Yield
Plasma or serum (per l.)	60,000	100%
Supernatant solution 1	56,000	93
Precipitate 2	45,000	75
Supernatant solution 3	36,000	60

Supernatant solution 1 is plasma or serum brought to 0.50 saturation with $(\text{NH}_4)_2\text{SO}_4$, dialyzed and dried from the frozen state. Precipitate 2 is the 0.70 saturated $(\text{NH}_4)_2\text{SO}_4$ precipitate dissolved, dialyzed and dried. Supernatant solution 3 is the final dried product from the preparation.

+5°C. and the degree of saturation increased to 0.70 by the addition of 13.5 g. C. P. $(\text{NH}_4)_2\text{SO}_4$ /100 ml. The precipitate is collected by centrifugation in the cold, dissolved in 100 ml. distilled water and dialyzed 18–20 hr. in Visking "No Jax" casing 29/32' against cold running tap or deionized water.

The clear dialysis residue (2 in Table II) is again brought to 0.50 saturation at +5°C. by the addition of an equal volume of saturated $(\text{NH}_4)_2\text{SO}_4$, adjusted to pH 3.75 with $N \text{ H}_2\text{SO}_4$, the precipitate removed by centrifugation in the cold and the supernatant solution neutralized to pH 7.0 with $N \text{ NaOH}$. The neutral solution is again dialyzed as above and dried from the frozen state (3 in Table II). The antifibrinolysin product obtained is about 85% pure on electrophoretic analysis.

POTENCY AND YIELD OF ANTIFIBRINOLYSIN

Theories concerning the origin of antifibrinolysin are pertinent to the problem of isolation of the enzyme inhibitor. These had their beginning with Achalme (14), who postulated that antiprotease is a protective

antibody formed against proteolytic ferments entering into the circulating blood. Meyer (15,16), and Kay and Lockwood (17), have reported an increased antifibrinolytic titre of serum following parenteral injections of trypsin in guinea pigs and dogs. This theory seems to be receiving impetus today despite Landsteiner's (4) indirect evidence, repeatedly confirmed, that antiprotease activity is a property of the albumin fraction. Also, there are no reports in the literature showing antifibrinolysin titres higher than 3-4 times normal (18,19) while antibody titres rise thousand-fold following the injection of antigen. If the titre of antifibrinolysin could be made to rise thousand-fold, a more potent source for its isolation could be prepared physiologically by pre-immunization with fibrinolysin or trypsin of the experimental animals whose blood serum is to be used in the biochemical procedures. However, since this has not been found to be especially helpful in increasing the yield obtained, normal plasma or serum are used.

Normal bovine plasma contains approximately 60 units antifibrinolysin/ml. The purified product assays 12-14 units/mg. and the yield is 3 g. Therefore, on a unit basis, this procedure recovers 60% of the available antifibrinolysin in a high degree of purity. Relative purification of partial products in the outlined procedure are shown in Table II.

RELATIONSHIP TO COAGULATION

Since Soy Antitrypsin has an effect on the clotting mechanism (20), we investigated the possibility that antifibrinolysin might also influence either the conversion of prothrombin to thrombin or the enzymic reaction changing fibrinogen to fibrin. Three possibilities were seen for inhibitory action in the first reaction above. Antifibrinolysin might react with prothrombin, accelerator globulin or thromboplastin. The following experiments were planned to determine the extent of any interference. Ten mg. (140 U.) antifibrinolysin were dissolved in 1 ml. 10,000 U./ml. prothrombin. After 15 min., at room temperature, the prothrombin was assayed using the modified 2-stage assay of Ware *et al.* (21). The prothrombin still assayed 10,000 U./ml.

Ten mg. antifibrinolysin were dissolved in 1 ml. purified concentrated accelerator globulin solution and allowed to stand at 25°C. for 15 min. The accelerator globulin solution was diluted 1-200 and used in the modified 2-stage assay of a 10,000 U./ml. control prothrombin solution. The accelerator globulin retained its activity.

Ten mg. antifibrinolysin were dissolved in 1 ml. incubation mixture,² the source of thromboplastin in the 2-stage prothrombin analysis, and allowed to remain at 25°C. for 15 min. This incubation mixture was then used in the 2-stage assay of the 10,000 U./ml. control prothrombin solution. The incubation mixture retained its full thromboplastic activity.

As an additional check on the conversion reaction a 3:1:1 prothrombin assay (22) was done, adding 10 mg. antifibrinolysin to the conversion reaction. Here again the prothrombin assayed 10,000 units/ml., indicating that the antifibrinolysin did not hinder the conversion. Antifibrinolysin in 10 mg./ml. concentration was compatible with a 0.3% fibrinogen solution used in the assay of thrombin and the results were in good accord with a control assay of the same thrombin.

Finally, dilute thrombin, having 1 mg. and 10 mg./ml. antifibrinolysin added to its solution, clotted the assay fibrinogen solution in the same time interval as the control thrombin.

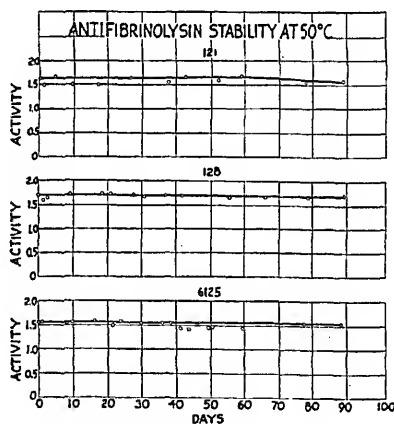


FIG. 1. These graphs show the unusual stability of dry antifibrinolysin (less than 1.0% moisture) over a period of 89 days at 50°C. at atmospheric pressure.

We feel that this series of experiments demonstrates that antifibrinolysin is not an antithrombin, antiprothrombin or antithromboplastin. It does not affect the activity of accelerator globulin or fibrinogen.

² Incubation mixture is prepared as follows: 3 parts diluted lung extract (21), 2 parts 15% purified acacia in saline, 2 parts 1% isotonic CaCl_2 , 1 part imidazole buffer, and 1 part physiological saline.

STABILITY OF ANTIFIBRINOLYSIN

Fig. 1 shows the results of accelerated stability studies with 3 typical preparations. The dry antifibrinolysin, containing less than 1% moisture, is stable for at least 90 days at 50°C. This indicates a stability of 3-4 yr. at temperatures near 25°C. Data on solutions of antifibrinolysin show that all activity is retained for several days at 25°C., 1-2 weeks at +3°C., at least 60 days at -7°C., and 2-4 months at -40°C.

PROPERTIES OF ANTIFIBRINOLYSIN

Antifibrinolysin has been prepared from human, equine and bovine sera and plasmas. It is a water-soluble, saline-soluble, nondialyzable protein antienzyme. It is not antithrombin, nor antiprothrombin. It is stable in the dry state and relatively stable in solution. It reacts with fibrinolysin neutralizing that enzyme, thus preventing the lysis of fibrin, fibrinogen and prothrombin. Antifibrinolysin has no effect on accelerator globulin.

SUMMARY

A method is presented for preparing antifibrinolysin from sera and plasmas in at least a 60% yield and in a high degree of purity. Data are given showing the stability and other properties of our preparations. A unit of antifibrinolysin is defined and our assay procedure explained and improved.

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Inhibition of Enzymatic Transphosphorylation by Alloxan and Ninhydrin in Tissue Extracts

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INTRODUCTION

Labes and Freisburger (1) have shown that alloxan destroys $-SH$ groups. Lehmann (2) found that addition of $-SH$ in the form of glutathione partially reverses the inhibition by alloxan of phosphoglucotase. Gemmill (3), apparently unaware of this work, has shown inhibition of glycogenolysis by alloxan. These facts, together with the observation (4) that pretreatment with anterior pituitary extract enhances hyperglycemia due to injection of alloxan into rabbits, suggested that hexokinase and phosphohexokinase might be inhibited by alloxan. Alloxan and ninhydrin (structurally related to alloxan) both oxidize the thiol groups of reduced glutathione in the blood of rodents (5,6); this indicates the possibility that ninhydrin might also inhibit enzymatic transphosphorylation.

The results of some experiments to test these possibilities are reported below.

METHODS

The methods in general are those of Cori and coworkers (7). The extracts, however, were prepared immediately before use from rat muscle or potato by mincing with, in the case of muscle, 3 volumes, and in the case of potato, 1.5 volumes of ice-cold distilled water in a Waring Blendor and filtering through a Whatman No. 41 filter paper on a Büchner funnel.

ATP¹ was prepared from rabbit, cat, or sheep muscle as the dibarium salt by Kerr's (8) method and used as the sodium salt.

¹ Abbreviations used: ATP = adenosine triphosphate. HMP = hexose monophosphate. P_0 = inorganic phosphate before hydrolysis. P_7 = inorganic phosphate after hydrolysis for 7 min. in 1 *N* HCl. $\Delta P_7 = P_7 - P_0$.

Ninhydrin and alloxan were used in the form of the monohydrate dissolved in distilled water. Such solutions in the concentrations used in this work have a pH as low as 3.50 for alloxan and 5.50 for ninhydrin. As alloxan is destroyed in neutral solutions (9), no attempt was made to neutralize the additions but the decrease in pH of the reaction mixture was controlled by additions of dilute HCl of appropriate pH. In experiments to determine the effect of cysteine on alloxan or ninhydrin, the inhibitor was allowed to act at room temperature on the reaction mixture for 5 min. before addition of cysteine (pH 6.70). In some experiments veronal sodium was added as a control for the extra buffering effect on the cysteine. This was later found to be unnecessary.

Reaction time was 40 min. and terminal pH 7.42–7.60. Volume of the reaction mixture was 1.60 cc., and all experiments were carried out at 37°C.

HMP was prepared by the method of Dubois and Potter (10) as barium HMP and used as sodium HMP.

Cori *et al.* (7), Meyerhof and Randall (11), and Broh-Kahn and Mirsky (12), have pointed out that apyrase of muscle is not completely inhibited by NaF, so that P_o increases in tissue extracts during incubation in the presence of ATP. This increase is not differentially affected to any extent by addition of alloxan, cysteine, or alloxan and cysteine (Table I).

TABLE I
Increase in P_o in Rat Muscle Extract

Number of experiments	P_o at 0 min.	Additions	P_o at 40 min.
2	131	HCl	140
		$2.86 \times 10^{-3} M$ alloxan	140
		$2.86 \times 10^{-3} M$ alloxan + $1 \times 10^{-2} M$ cysteine	144
		$1 \times 10^{-2} M$ cysteine	140

Because of the increase in P_o , transfer of phosphate from ATP to glucose or HMP was measured by the decrease in P_7 after incubation, and not by the decrease in ΔP_7 .

Inorganic phosphate was measured by the method of Fiske and SubbaRow (13) on a photoelectric absorptiometer. Reaction mixture containing ninhydrin and cysteine had a strong red color. This, however, was destroyed by trichloroacetic acid during precipitation of protein and did not interfere with phosphate determination.

RESULTS

Under the conditions described, concentrations of $1.25 \times 10^{-3} M$ to $5 \times 10^{-3} M$ alloxan brought about 25–100% inhibition, and $5 \times 10^{-3} M$ ninhydrin 80% inhibition

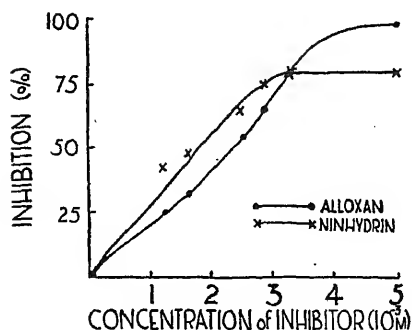


FIG. 1. Inhibition of muscle hexokinase by alloxan and ninhydrin.

of muscle hexokinase (Fig. 1). $2.86 \times 10^{-3} M$ alloxan also substantially inhibited muscle phosphohexokinase (Table II).

Concentrations up to $2.86 \times 10^{-3} M$ cysteine had little effect on the inhibition of muscle hexokinase by $2.86 \times 10^{-3} M$ alloxan. Such concentrations of cysteine, however, produced a marked increase in phosphate transfer in the absence of inhibitor (Fig. 2).

$5 \times 10^{-3} M$ and $1 \times 10^{-2} M$ cysteine, however, completely released the inhibition by $2.86 \times 10^{-3} M$ alloxan (Table II) and further increased phosphate transfer (Fig. 2).

Addition of 380 γ of insulin/cc. of reaction mixture had no effect on the inhibition by $2.86 \times 10^{-3} M$ alloxan (Table II).

$5 \times 10^{-3} M$ cysteine had little effect on the inhibition of muscle hexokinase by $2.5 \times 10^{-3} M$ ninhydrin, while $1 \times 10^{-2} M$ cysteine effected a partial release (Table II).

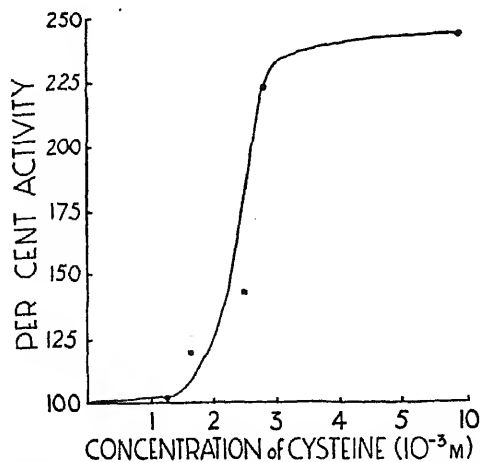


FIG. 2. Acceleration of muscle hexokinase by cysteine. The activity of extracts without addition of cysteine is represented as 100%.

TABLE II
*Effect of Alloxan, Ninhydrin, and Various Additions on Transfer of
 Phosphate from ATP to Glucose or HMP*

Tissue	No. of experiments ^a	Substrate	P _i at 0 min.	Additions	P _i at 40 min.	P transfer
Rat muscle	4	HMP	380.0	HCl	337.5	42.5
				2.86×10 ⁻³ M alloxan	367.5	12.5
Rat muscle	8	Glucose	402.0	HCl	342.0	60.0
				2.86×10 ⁻³ M alloxan	379.0	23.0
				2.86×10 ⁻³ M alloxan + 380 γ insulin/cc.	381.0	21.0
Rat muscle	2	Glucose	390.0	HCl	320.0	70.0
				2.86×10 ⁻³ M alloxan	363.0	27.0
				2.86×10 ⁻³ M alloxan + 1.25×10 ⁻³ M cysteine	363.0	27.0
				2.86×10 ⁻³ M alloxan + 1.67×10 ⁻³ M cysteine	362.0	28.0
				2.86×10 ⁻³ M alloxan + 2.86×10 ⁻³ M cysteine	352.0	38.0
				2.86×10 ⁻³ M alloxan + 5×10 ⁻³ M cysteine	323.0	67.0
				2.86×10 ⁻³ M alloxan + 1×10 ⁻² M cysteine	321.0	69.0
Rat muscle	4	Glucose	395.0	HCl	317.5	77.5
				2.5×10 ⁻³ M ninhydrin	365.0	30.0
				2.5×10 ⁻³ M ninhydrin + 5×10 ⁻³ M cysteine	362.0	33.0
				2.5×10 ⁻³ M ninhydrin + 1×10 ⁻² M cysteine	339.0	56.0
Potato	4	Glucose	360.0	HCl	310.0	50.0
				2.86×10 ⁻³ M alloxan	312.0	48.0

^a Four experiments indicate two different extracts, each done in duplicate. Duplicates were usually identical and never varied by more than 10 γ.

Potato hexokinase was not inhibited by $2.86 \times 10^{-3} M$ alloxan in the dilution of enzyme used. Potato tissue extracted with more than 1.5 cc. of water/g. of tissue showed such marked apyrase, or possibly hexosemonophosphatase, activity that a hexokinase effect could not be detected. Efforts are being made to purify this hexokinase and the effect of alloxan will be further studied.

DISCUSSION

From the results, it is apparent that more than one molecule of cysteine is needed to release the inhibition of muscle hexokinase by one molecule of alloxan or by one molecule of ninhydrin. Lieben and Edel (14) have pointed out that, although alloxan combines most readily with thiol groups of proteins and amino acids, it also is capable of decarboxylation and desamination of α -amino acids (Strecker reaction). It is possible that alloxan has some effect on α -NH₂ groups in hexokinase. In support of this, considerably more cysteine is required to release partially, ninhydrin inhibition of hexokinase than to release alloxan inhibition completely, and ninhydrin, while able to combine with thiol groups, has many times the potency of alloxan as a decarboxylating and desaminating agent (15).

Lehmann (2) has also considered the possibility of alloxan forming a complex with some groups other than thiol groups in phosphoglucumutase on the basis of his finding that $-SH$ glutathione only partially released alloxan inhibition of the enzyme. Lehmann also observed an activation by $-SH$ glutathione, similar to that reported above for muscle hexokinase, of phosphoglucumutase. Hopkins *et al.* (16) observed a similar acceleration of succinic dehydrogenase, and only a partial release of alloxan inhibition, by $-SH$ glutathione. α -Glycerophosphate dehydrogenase was not inhibited by alloxan.

It is interesting to note that a purified enzyme such as crystalline muscle phosphorylase is strongly activated by cysteine (17), as is crude muscle hexokinase. Thiol groups, however, appear to have no stimulatory effect on the activity of crystalline yeast hexokinase (18).

The absence of inhibition of potato hexokinase by alloxan is paralleled by other observations on hexokinase of non-animal origin. Price, Cori and Colowick (19) found that anterior pituitary extract, which inhibits muscle hexokinase, has no inhibitory effect on yeast hexokinase and Meyerhof and Randall (11) report no inhibition of the same enzyme with adrenochrome, which inhibits brain hexokinase. Further elucidation of the differences between plant and animal hexokinases will probably await the purification of the enzymes.

SUMMARY

$5 \times 10^{-3} M$ alloxan inhibits muscle hexokinase 100% and $5 \times 10^{-3} M$ ninhydrin inhibits 80%. Muscle phosphohexokinase is substantially inhibited by $2.86 \times 10^{-3} M$ alloxan. $5 \times 10^{-3} M$ cysteine completely released the inhibition of muscle hexokinase by $2.86 \times 10^{-3} M$ alloxan, and such concentrations of cysteine bring about a considerable increase in phosphate transfer in the absence of inhibitor. $1 \times 10^{-2} M$ cysteine effected only a partial release of inhibition of muscle hexokinase by $2.5 \times 10^{-3} M$ ninhydrin.

Potato hexokinase was not inhibited by $2.86 \times 10^{-3} M$ alloxan.

More than one molecule of cysteine is needed to release the inhibition of muscle hexokinase by one molecule of alloxan. This, together with the observations that far more cysteine is needed to release inhibition by ninhydrin, and that ninhydrin is more potent as a desaminating and decarboxylating agent than alloxan, suggests that inhibition by these compounds is effected by more than thiol destruction alone.

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On the Mechanism of Enzyme Action. XXXIV. The Influence of a Pigment from *Fusarium solani* D₂ Purple (Solanione) on the Composition of Fats Formed in *Fusaria*

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INTRODUCTION

In previous investigations from this laboratory it was observed that *Fusaria* possess a fatty acid dehydrogenase (1), and that when solanione, a pigment present in *Fusarium solani* D₂ purple (FsD), is added to a growing culture of the non-pigment producer *Fusarium lini* Bolley (FlB), there is a decrease in mycelial weights, and the carbohydrate conversion factor is greatly lowered (2). *Fusarium lycoperisci* (Flyco), on the other hand, produces fat and pigments to a larger extent when grown on Raulin-Thom media containing increasing amounts of glucose. Similarly, the mold FsD was shown to have a maximum lipide production accompanied by maximum pigment accumulation when the glucose concentration amounts to 5% in the Czapek-Dox media.

These observations indicated a relationship between the mechanism of fat formation and the possible interaction of an added pigment in dehydrogenations taking place in the course of the carbohydrate conversion.

While there are many instances recorded in the literature (3) as to the effect of the environment upon the production and characteristics of fats of different species (4), only a few observations were made in relation to the possible influence of natural pigments on the production of certain metabolic constituents.

It is interesting to quote, for instance, the comparative studies undertaken by Lovern (5) on the relation between the type of fat produced by different species of algae and their color grouping.

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There seems to exist a definite connection between the unsaturation of the fats of the species studied and their color. Lovern reported that, for example, the green algae contain predominantly unsaturated acids having 16-18 carbon atoms with a small proportion of C_{20} - C_{22} acids, the degree of unsaturation of the first group being unusually high in the average. A red alga examined contained C_{20} acids as major components.

Since, from the above mentioned work from this laboratory, it would appear that the addition of solanione (6) is quantitatively related to the conversion of carbohydrate to fat, an investigation as to whether the *composition* of the fat produced by FIB and Flyco is altered by the presence of the pigment was suggested by Dr. Nord. If so, then its effect on a hydrogen transport system would be demonstrated.

EXPERIMENTAL

The *Fusarium* cultures used and the cultural conditions applied were the same in the present experiments as those mentioned in the previous communications. FIB was grown on a Raulin-Thom medium containing 2.5% glucose. A culture of Flyco was obtained also, through the courtesy of Dr. C. D. Sherbakoff, Knoxville, Tenn. This was maintained and transferred in a potato dextrose-agar medium (39 g./l. of tap water) and grown on Raulin-Thom medium containing 5% glucose.

Parallel series of FIB and Flyco each were prepared in the presence and absence of solanione. The pigment in pure state, dissolved in acetone (1 mg./cc.), was added to each flask from a burette prior to sterilization. The concentration of solanione in this series was 1 mg./l. of medium.

PROCEDURES

A FIB series was grown for 21 days in the absence of pigment. Simultaneously a FIB series in the presence of pigment grew for the same length of time. All the conditions, with the exception of the addition of pigment, were the same for both series. The Flyco series grew for 25 days, and strongly pigmented mats were obtained, using the stock cultures from potato-dextrose-agar (7). The mats of FIB and Flyco were filtered and washed with water. They were then dried over paper in the air with the aid of an electric fan and ground to 40 mesh. The ground material was placed in a vacuum desiccator for 24 hr., weighed, and in all cases extracted in a Soxhlet or McFarland apparatus (8) for 50 hr., using low boiling (30° - 60° C.) petroleum ether as solvent. The petroleum ether extracts were filtered through a sintered glass funnel and the fats collected by distilling the solvent under reduced pressure. At the end of this operation the fats were preserved in a nitrogen atmosphere and kept in the cold.

EXPERIMENTS WITH FLYCO

Flyco Grown in the Absence of Solanione. The average mat weight amounted to 9.8 g. The amount of fat obtained per mat was 1 g.; thus, the content of fat in the mycelium was 10%. The reddish brown semisolid fat possessed an iodine number of 80-81 (Hanus).

Phospholipides. One g. of the crude fat was dissolved in 5 cc. of ether, and acetone was added in an equal volume. The nonappearance of a precipitate or turbidity indicated the absence of phospholipides.

Saponification. A 6% solution by weight of KOH in alcohol was employed (9) for the saponification of Flyco fat. Thirty g. of fat was treated with 150 g. of this solution and refluxed for 3 hr. At the end of the saponification, the soap solution was concentrated to half its volume *in vacuo* and the same volume of water added.

Unsaponifiable Matter. The resulting volume of soap solution was extracted with ether by means of a continuous extractor as proposed by Hilditch (9). The ethereal solution was evaporated under reduced pressure to half its volume and, after addition of the same amount of water, extracted again in the same manner. The ethereal solution obtained was washed with water, the ether layer separated and dried over anhydrous sodium sulfate. After standing for 24 hr., the solution was filtered and the filtrate distilled *in vacuo*. The residue was then dried to constant weight at 100°C. The unsaponifiable matter amounted to 5.84% of the total fat. It has been reported as consisting mainly of ergosterol (10).

Total Fatty Acids Mixture. The soap solutions obtained after the two successive saponifications, together with the washings, were acidified to Congo red with HCl, a current of nitrogen being passed through the liquid during the addition of the acid to protect the acids from oxidation. The fatty acids were then extracted 5 times with ether, and the ether extract washed with water until the washings gave a negative chloride reaction.

The ethereal solution was dried over anhydrous Na_2SO_4 and placed in the ice box over night. The solution was filtered and the solvent evaporated *in vacuo*. The mixture of fatty acids was then dried under reduced pressure. The total fatty acids amounted to 86% of the fat and had an iodine number of 83 (Hanus).

Fractionation of the Mixed Fatty Acids. For this purpose the low temperature crystallization procedure as proposed by Earle and Milner (11) was applied. By this method it was possible, maintaining conditions very closely, to obtain satisfactory duplicate results. The saturated fraction obtained by this means was practically colorless and possessed a low iodine number. After applying a correction for unsaturated acids, estimated as oleic, the total fatty acid mixture was found to consist of 66.2% of liquid acids and 33.4% of saturated acids. No unsaturated solid acid was present in the fat.

Qualitative Composition of the Liquid Fraction. The liquid fatty acids were submitted to bromination (12). The fatty acids were dissolved in anhydrous ether (10% solution). The mixture was cooled below $-10^\circ\text{C}.$, and bromine added drop by drop from a separatory funnel. The mixture was constantly shaken. The addition was stopped when the solution acquired a lasting reddish color. At this point, no precipitation had occurred, indicating the absence of linolenic acid. The brominated mixture, after standing for 20 min. at the given temperature, was washed with a 2% solution of $\text{Na}_2\text{S}_2\text{O}_3$ in a separatory funnel to eliminate the excess of halogen, and then with water. The ethereal solution was separated and dried over anhydrous Na_2SO_4 .

The solution was then filtered and the ether removed *in vacuo*. The ether-soluble bromides were boiled under reflux with petroleum ether ($30^\circ\text{--}60^\circ\text{C}.$) for 30 min. A precipitate came out slowly upon cooling, indicating the presence of linoleic acid. The

dibromide remaining in the ethereal solution, together with any possible fraction of soluble higher bromides, were not further investigated.

Flyco Grown in the Presence of Solanione. The average weight of a Flyco mat grown in the presence of the pigment was the same as in the control series (9.8 g.). The fat per dried mycelium amounted to 10.4%, differing only slightly from the control. The iodine number was 79–80. The fat was treated in the same manner as for the series prepared in the absence of pigment. The total fatty acids, determined as previously mentioned, gave a figure of 84% in the crude fat.

A further separation of liquid and solid fractions from the total fatty acids showed that the ratio: unsaturated/saturated acid was 2:1 (65.1% liquid and 32.5% saturated acids), as compared with a ratio 1.96:1 for the fat produced under normal conditions.

EXPERIMENTS WITH FLB

FLB Grown in the Absence of Pigment. The average mat weight amounted to 4.83 g. The fat content in the dried mycelium was 5.63%.

A detailed study on FLB carried out in this laboratory (10) has shown that the fat possesses an iodine number of 84 and that the liquid fatty acids consist mainly of oleic acid 53.5% and linoleic acid 47.1%.

FLB Grown in the Presence of Solanione. The average weight of a dried mat was 3.01 g. The fat content in the dried mycelium amounted to 5.26%. The qualitative composition of the liquid fatty acids was the same as reported for the fat produced under normal conditions. It had, however, an iodine number of 138. To corroborate this last finding and compare the unsaturation of the fats produced in the presence and absence of solanione, the spectrophotometric method after alkali isomerization was utilized (13).

Comparative Figures of FLB Data Obtained in the Presence and Absence of Solanione. There was a 30% reduction in the weight of the dried mycelia of the mold grown in the presence of the pigment. There is, however, an increase of 54 units in iodine number of the fat as a result of the addition of pigment and a slight decrease in the relative amount of fat produced, namely 0.4%.

Since the two fats were produced from the same mold under identical conditions, the only difference being the addition of the pigment, it was thought desirable to compare the spectrophotometric curves developed from ultraviolet absorption measurements obtained in the range of

246–310 $m\mu$. The resulting curves obtained with a Beckman spectrophotometer are shown in Fig. 1.

It is to be noted that the two absorption curves are practically identical in the range of 265–310 $m\mu$, probably due to the presence of ergosterol (14). However, there is a significant deviation between 246 and 265 $m\mu$, indicating that a partial change in the quantitative composition of the fat occurred.

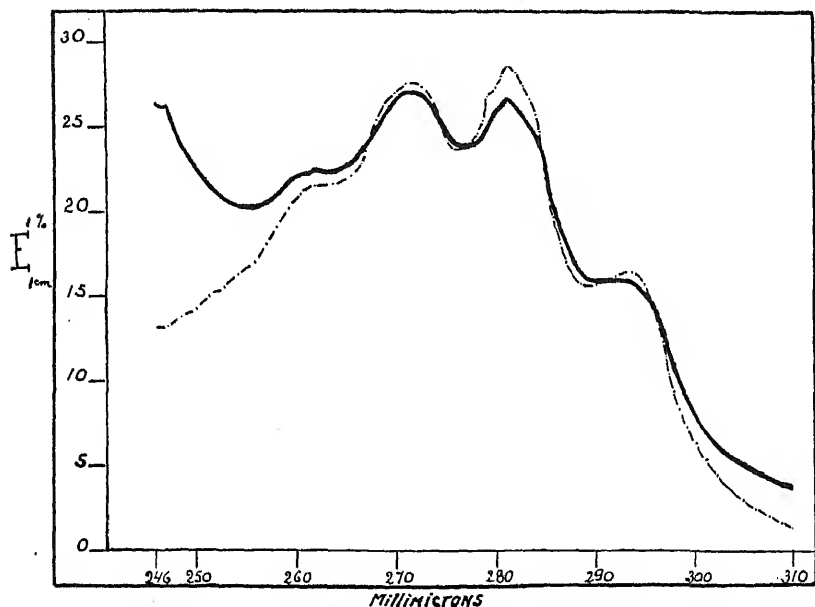


FIG. 1. Spectrophotometric curves of fats from *Fusarium lini* produced normally and under the influence of solanione. — Normally produced fat. - - - - - Produced under solanione effect.

Isooctane of high purity was employed as solvent (15). It is also of interest to note that, upon dissolving the respective fats in this solvent, the FIB fat produced in the absence of pigment gave a difficultly soluble dispersion which was dissolved only after further dilutions. The fat produced in the presence of solanione was readily soluble in isooctane.

Comparison of Spectrophotometric Values after Alkali Isomerization. To understand the increase in iodine number of the fat obtained in the

presence of solanione, it was necessary to discover whether the relative amount of linoleic acid present in the fat had increased. The possibility existed that *linolenic* acid, not detected by the bromination method, had been formed also. An iodine number as high as 138 must be understood to have resulted from the presence of an unsaturated acid with a higher iodine value than oleic (90). The constituents of F1B fat produced under normal conditions, which could absorb iodine, were oleic acid, linoleic acid, and sterol (2), the quantity of the latter remaining unchanged with or without added solanione. Still, there was a possibility that linolenic acid with a high iodine number of 274.1, and present in the normally produced fat in even smaller amounts, had not been detected by the bromination procedure.

Once this last possibility was eliminated, the increase in iodine number could only be understood either on the basis of newly produced linolenic acid, caused by the interference of the solanione in the course of desaturation, or simply because of an increase in the relative amount of linoleic acid with a consequent decrease of oleic acid.

To establish the possibility of the presence of linolenic acid in the two fats, measurement of the ultraviolet absorption at $268\text{ m}\mu$ was carried out on the soaps after alkali isomerization. There was no characteristic absorption at that wave length. The determination was carried out on both fats using the same conditions. Thus, the possibility of the presence of the triethenoid acid was eliminated. These measurements corroborate the previously mentioned chemical data and support the conclusion that the increase in iodine number can only be due to an increase in linoleic acid.

COMMENTS

An effect of the FsD pigment (solanione) in a low concentration of 1 mg./l. of medium on the growth of a highly pigmented mold such as *Flyco*, in the light of our experimental data, is not to be taken into consideration. The pigments originally present in the mold or compounds of probably related structures exist in such abundance as compared to the quantity added to the medium, that the addition has no measurable influence on the growth. As to the effect of the pigments on the carbohydrate conversion into fat, the fact that no changes were noticed in its component fatty acids indicates that, if pigments were of some influence on the enzymatic mechanism of fatty acid formation,

it might have taken place as a result of the original pigment produced by the mold itself.

With the nonpigmented mold F1B chosen for this experiment the case is different. There was a strong decrease in the overall growth of the organism due to the addition of solanione and a pronounced effect on the composition of the fat produced under those conditions. That a more extensive desaturation took place as a result of the addition of pigment, can be seen by the increase in the absorption of iodine. The consolidated results are recorded in Table I.

TABLE I

Species	Pigment producer <i>Fusarium lycopersici</i>		Non-pigment producer <i>Fusarium lini</i> Bolley	
Medium employed	Raulin-Thom			
	5% Glucose		2.5% Glucose	
Period of growth	25 days		21 days	
Series	Control (with- out pigment)	Pigment added	Control (with- out pigment)	Pigment added
Dried mycelium g./l.	9.8	9.8	4.82	3.01
Total lipide in myce- lium per cent	10.4	10	5.63	5.26
Iodine number	80-81	79-80	84	138

That the excess of desaturation should be considered as an increase in linoleic acid, can be deduced from the qualitative studies in both fats as well as from the absence of linolenic acid in the alkaline soaps.

SUMMARY

1. Solanione, a pigment present in *Fusarium solani* D₂ purple, has no appreciable effect upon the growth of, and the type of fat produced by, the mold *Fusarium lycopersici*, which can be grown as a pigment producer.

2. Solanione, as shown previously, has a marked effect on the growth of the non-pigmented *Fusarium lini* B. The pigment under consider-

ation is the cause of the change in the fat metabolism of the mold, namely, the desaturation of the fatty acid constituents, as shown by the difference in iodine absorption.

3. The addition of solanione increases the desaturation of fats produced by *Fusarium lini* Bolley and seems to affect the hydrogen transport system present in this mold.

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On the Mechanism of Enzyme Action. XXXV. A Relationship between Ergosterol Formation in *Lentinus lepideus* and Lignification

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INTRODUCTION

The formation of sterols by microorganisms is well established. In yeasts and molds, the sterol, ergosterol, often forms a high proportion of the unsaponifiable fraction. Although ergosterol has been detected in small amounts in both animals and plants, it is generally conceded that this substance is the principal sterol present in the filamentous fungi (1).

Thus, ergosterol has been isolated from various *Aspergilli* and *Penicillia*. For example, *Aspergillus fischeri* (1) and *Penicillium puberulum* (2) have the ability of synthesizing ergosterol when grown on synthetic media containing glucose as the sole source of carbon. Most recently, investigations in this laboratory (3) have established for the first time the formation of ergosterol by members of the genus *Fusarium*, namely, *F. lini* Bolley, *F. lycopersici*, and *F. solani* D₂ purple.

In a series of publications, J. Zellner was able to isolate ergosterol from many of the higher fungi, including several of the wood-rotting species, such as *Amanita muscaria* (4a), *Armillaria mellea* (4b) and *Polyporus sulphureus* (4c). The interest of this laboratory in the mechanism of lignin formation (5) prompted the continuation of investigations into the metabolism of certain of these fungi.

During the course of such investigation, the presence of a sterol was detected in the mycelium of the basidiomycete, *Lentinus lepideus* (Lelep),² an organism causing brown rot in wood. This fungus, known to give rise to the aromatic ester, methyl-*p*-methoxycinnamate, when

¹ Communication No. 170. This study was carried out with the aid of a grant from the Office of Naval Research.

² Metabolic products of *Lentinus squamosus* (*lepideus*) were investigated by Zellner and Zikmunda (4c), but no indication is given as to the nature of the sterol fraction.

grown on cellulose, glucose, xylose, or Scots pine wood, was shown to form this ester from the cellulosic fraction of the wood rather than the lignin (6). Evidence was presented which demonstrated that the C_2 compound, acetaldehyde, functions as a key intermediate in this biosynthesis (7).

The isolation, identification, and mechanism of formation of the sterol, ergosterol, by *Lelep*, is the subject of this report.

EXPERIMENTAL

The organism, originally received through the courtesy of Dr. W. J. Robbins of the New York Botanical Garden, was cultivated on a medium consisting of:

Glucose	20.0 g.
Neopeptone	1.0 g.
KH_2PO_4	1.5 g.
$MgSO_4 \cdot 7H_2O$	0.5 g.
Thiamine hydrochloride	2 mg.
Tap water to	1 l.

Stock cultures were maintained on the above medium, supplemented with 20 g. of agar, and incubated in the dark at $27^\circ C$.

Isolation of the Sterol. One liter portions of the above medium, contained in each of several 3-liter Fernbach flasks, were sterilized by autoclaving for 20 min. at 15 lbs. pressure. Each flask was inoculated with a two week-old culture of *Lelep*, grown on 50 ml. of the medium in 125 ml. Erlenmeyer flasks. After 2-3 months of incubation (depending on the rate of glucose consumption), the resulting mats were filtered off, washed with distilled water and dried. The ground mats were extracted with chloroform in a Soxhlet apparatus for 48 hr. The solvent was removed by distillation, and the fatty residue saponified twice with 4% alcoholic KOH, as in previous investigations from this laboratory (8).

The ethereal solution of the unsaponifiable fraction was evaporated to dryness, and the residue recrystallized several times from alcohol-benzene (2:1) and from absolute ethanol. The resulting crystalline material melted at $158-60^\circ C$. and responded strongly to the color reactions characteristic of sterols.

Characterization of the Sterol. A mixed melting point of the sterol isolated from *Lelep* with an authentic sample of ergosterol showed no depression. Accordingly, the data obtained for this sterol were compared with those reported for ergosterol.

	Sterol from <i>Lelep</i>	Ergosterol
Melting Point	$158-60^\circ C$.	$160-1^\circ C$. (1)
Absorption maxima	260, 271, 282, 293.5 $m\mu$	260, 270, 282, 293.5 $m\mu$ (1)
Acetate, M.P.	$172-3^\circ C$.	$173-5^\circ C$. (1)
Benzoate, M.P.	$168-9^\circ C$.	$168-9^\circ C$. (1)

In addition, the sterol from *Lelep* reacted to the Salkowski, Liebermann-Burchard, Tortelli-Jaffé, chloral hydrate, trichloroacetic acid, *etc.*, color reactions, in a manner identical with that reported for ergosterol (9).

From these data, it is concluded that the sterol produced by Lelep has been identified as ergosterol.

In further experiments, 20 ml. of 95% ethyl alcohol were added aseptically to one liter portions of the sterilized peptone-salt solution with glucose omitted. The flasks were inoculated with mats of Lelep, and after a period of incubation the mycelia were filtered, *etc.*, and the unsaponifiable fraction isolated as previously. A crystalline material was obtained which was proven by melting point, mixed melting point, absorption spectrum, *etc.*, to be identical with the sterol obtained on the glucose medium.

Quantitative Estimation of Sterol Production. The amount of sterol present in microorganisms is said to vary between 0.1 and 1.7% (11). For completeness of information, quantitative estimations of the total sterol content of fully developed Lelep mats were performed. The colorimetric method employing the blue color produced with 90% trichloroacetic acid (10) was replaced by that utilizing the Liebermann-Burchard reaction, as in use in this laboratory (3).

After 3 months of cultivation on the 2% glucose medium, the fully grown mats of Lelep were filtered off, and then, dried, ground, and weighed samples of these mycelia were extracted with chloroform for 24 hr. in Soxhlet extractors. Aliquots of the resulting solution were subjected to the Liebermann-Burchard reaction; the intensity of color development was measured with the Evelyn colorimeter and compared with standard values. The following are results obtained from 4 different samples of mycelia:

3.21 mg. sterol/g. mycelium	
3.25 mg. " "	
3.21 mg. " "	
3.31 mg. " "	

Accordingly, it is estimated that sterol is present to the extent of approximately 0.3% in the fully developed mat of Lelep.

RESULTS AND DISCUSSION

Evidence exists that ergosterol is synthesized by microorganisms from some C₂ compound (11). Thus, Halden and coworkers (12) succeeded in increasing the sterol content of yeast about 60-fold, by keeping the yeast suspension in thin layers on agar containing sucrose, and supplying oxygen and alcohol vapors. Moreover, Sonderhoff and Thomas (13) demonstrated that, when utilizing trideuteroacetic acid as substrate, yeast cells accumulated considerable deuterium in the unsaponifiable fraction, thereby suggesting the synthesis of sterol from the acetate. Finally, Maguigan and Walker (14), on the basis of their

experiments, concluded that acetate can provide the necessary carbon for sterol synthesis by yeast.

The previous observation (7) that *Lelep* causes an alcoholic fermentation of glucose as well as xylose, and that the alcohol so formed was quickly dehydrogenated, indicated that the aromatic ester, methyl-*p*-methoxycinnamate, formed by this organism, had been synthesized from a common reactive breakdown product arising from both carbohydrates. These findings made it appear that the acetaldehyde formed as a result of the dehydrogenation of ethyl alcohol could be the transient product which is then enzymatically converted into the ester. The experiments presented gave conclusive evidence that the C_2 compound which serves as a key intermediate in this enzymatic synthesis is acetaldehyde, and that this substance constitutes the connecting link in the catabolism of carbohydrate and the synthesis of the ester which, in turn, is closely related to certain lignin breakdown products.

Moreover, in a study of fat and sterol formation in *Fusaria* (3), it was found that xylose is a much more suitable substrate for conversion into lipides than the hexoses, glucose and fructose. This was attributed to the fact that twice as much acetaldehyde is obtainable from the hexoses in comparison with that derived from the same amount of xylose, and that the enzymatic synthesis of fat and sterol from the aldehyde, or acetic acid, progresses slower than the decarboxylation of pyruvic acid to the aldehyde.

These observations on unrelated organisms suggest the probability that acetaldehyde represents an essential C_2 intermediate connecting the catabolism of carbohydrate and the anabolism of lipide by fungi. Accordingly, it was thought conceivable that this substance might play an important role in the formation of sterol by *Lelep*, since it also seems to serve as a connecting link in the carbohydrate-lignin transition (7).

The identity of the sterols obtained from glucose and alcohol-containing media then corroborates the previously reported (7) fermentation of glucose by *Lelep* to ethyl alcohol, and suggests that some C_2 compound does indeed take part in the formation of sterol by this organism.

To investigate the possibility that this substance might be acetaldehyde, an attempt was made to trap the acetaldehyde formed by dehydrogenation of the ethyl alcohol of the medium, employing dimedon

(dimethyldihydroresorcinol), the experiment to consist in a quantitative comparison of the sterol formed on the dimedon-supplemented and the unsupplemented media by periodic analyses. The medium employed was that used in the isolation of the sterol from *Lelep* grown on alcohol, and in the interception experiments, 0.5 g. of dimedon was added per l.

Fifty ml. portions of the sterilized salt-peptone medium, to which was added aseptically 1 ml. of ethyl alcohol, were inoculated with 1 cc. of spore-mycelial suspension of *Lelep*. The flasks were incubated at 27°C., and their contents analyzed at the end of 0, 2, 3, 4, and 6 weeks.

Analytical Procedures

(1) *Mycelial Weights*. The contents of from 4 to 8 flasks were filtered through tared alundum crucibles; these were dried at 60°C. for 48 hr. and reweighed.

(2) *Alcohol Content of Medium*. An appropriate volume of the filtrate obtained above was analyzed for its alcohol content in the usual way.

(3) *Sterol Content of Mycelium*. The dried and weighed mycelia were extracted in Soxhlets with chloroform for 24 hr.; the sterol content of the extract was determined colorimetrically, as above.

(4) *pH of Medium*. The pH's of the above filtrate were determined using a Cambridge electron-ray pH meter.

TABLE I

Time (weeks)	Mycelial weight (mg./100 ml.)	Alcohol content of medium (mg./ml.)	Sterol content of mycelium (mg./g.)	pH of medium
Alcohol Medium, Unsupplemented				
0	0.0	9.9	0.0	5.7
2	8.8	8.8	Trace	4.7
3	11.9	6.8	1.9	4.5
4	43.4	4.5	1.8	4.3
6	144.4	1.4	1.1	4.2
Alcohol Medium, Supplemented with Dimedon				
0	0.0	10.5	0.0	4.8
2	9.8	9.0	Trace	4.6
3	13.6	7.4	1.6	4.5
4	59.1	4.9	1.5	4.3
6	153.0	1.1	1.9	3.9

Table I shows that the sterol contents of the mycelia formed on both the dimedon-supplemented and unsupplemented media are equivalent. However, the concentration of dimedon employed (0.5 g./l.) is very low in comparison to the amount which would be required to trap all the acetaldehyde available from the alcohol present, thereby enabling most of the acetaldehyde to engage in its normal metabolic functions, as is evident from the progressive equivalency of the mycelial weights in the two parallel experiments.

It was, therefore, of importance to find the maximal concentration of dimedon which would permit growth of the organism on this medium, and to determine whether or not sterol was formed at this level of trapping agent.

Hence, to separate portions of the usual peptone-salt medium, increasing amounts of dimedon were added, to correspond to the following concentrations:

Series	Conc. dimedon/l. g.
1	0.5
2	1.0
3	2.0
4	4.0

Fifty ml. portions of these media, each containing 1 ml. ethyl alcohol, were inoculated as previously and incubated for 4 weeks, at which time their contents were filtered through tared crucibles. These were dried, washed with ether to remove the crystalline compound, previously identified as the acetaldehyde-dimedon addition complex (7), redried, weighed, and extracted with chloroform in Soxhlets. The resultant extracts were concentrated by evaporation, and tested for sterol by the Liebermann-Burchard reaction, with the results given in Table II.

TABLE II

Series	Mycelial weight (mg./100 ml.)	Sterol test
1	55.3	Positive
2	22.9	Positive
3	13.7	Positive
4	5.8	Trace

COMMENTS

The continued production of sterol at the higher concentration levels of dimedon would at first appear to indicate that acetaldehyde, since it was trapped by the dimedon, was not a precursor in the genesis

of the sterol. However, it must again be emphasized that the concentration of dimedon in these experiments is still meager in relation to the stoichiometrically required amount. Therefore, while it is impossible, under our experimental conditions, to trap all the aldehyde obtainable by dehydrogenation of the alcohol of the medium, yet, the inverse ratio found between dimedon concentration and mycelial weight, as is evident from Table II, still suggests that acetaldehyde is the C_2 compound which has a key role in the conversion of carbohydrate to various end products by Lelep, thereby amplifying the previously reported (7) indispensability of this labile intermediate in the formation of methyl-*p*-methoxy cinnamate by this organism.

These results, therefore, afford additional evidence of the paramount role of acetaldehyde in enzymatic conversions in which carbohydrate is transformed *via* multi-phase pathways into the various end products of metabolism. While this does not imply that the formation of lignin from carbohydrate *via* acetaldehyde requires the actual intervention of the fungus, Lelep, it does indicate the probability that enzyme systems similar to those present in this organism are instrumental in the process of lignification.

SUMMARY

1. The wood-destroying organism, *Lentinus lepideus*, produces ergosterol from glucose and ethyl alcohol.
2. Sterol is present to the extent of about 0.3% of the weight of the fully developed mat.
3. Further evidence is presented which indicates that acetaldehyde plays an essential role in the overall carbohydrate metabolism of the organism.

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LETTER TO THE EDITORS

Magnesium—Potassium Antagonism

INTRODUCTION

Dogs fed a diet low in potassium and lacking certain members of the B complex have a reduced serum potassium and develop a curare-like paralysis (1,2,3,4). The resemblance of this paralysis to that produced in dogs by an excess of magnesium suggested a possible relationship between potassium and magnesium (5).

EXPERIMENTAL

Six dogs, from 7–12 kg. in weight, were given magnesium sulfate parenterally in doses ranging from 0.25 to 2.2 g. per kilo. The first dog received subcutaneously 2.2 g./kg. of a 25% aqueous solution of magnesium sulfate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$). This resulted in the usual picture of nausea, vomiting, respiratory distress with reduced rate but with the diaphragm in action, and, finally, in a flaccid paralysis with loss of all the deep reflexes. Accompanying these changes there was a drop in serum potassium from 20.0 to 13 mg.-% or a decrease of 35%. The second dog received intravenously 0.5 g./kg. of a 50% solution of the anhydrous material¹ *via* the jugular vein. This animal died almost immediately. Blood obtained posthumously, about 10 min. elapsing after the injection, showed a drop in serum potassium from 21.5 to 18 mg.-%, or a decrease of 16%. The third dog received 0.3 g./kg. of the same solution in the saphenous vein of the leg. This dog died in a paralyzed state after approximately 0.5 hr. The serum potassium at the time of death showed a decrease from 23.0 to 12.5 mg.-%, or 46%.

A sufficient amount of a 10% solution of anhydrous magnesium sulfate to supply from 0.3 to 0.4 g. MgSO_4 /kg. was injected into the saphenous veins of the last 3 dogs without a fatality. The serum electrolytes, magnesium, calcium, sodium, and potassium² were determined and correlated with the clinical condition as paralysis devel-

¹ Prepared commercially by the Abbott Laboratories.

² Methods: Serum calcium was assayed by a modification of the method described by Smith (6), and the magnesium by that of Simonsen, Westover and Wertman (7). Both were carried out in triplicate with excellent concordance. The sodium and potassium assays were made by means of the flame photometer, Model 52 A (The Perkin-Elmer Corp.), using the internal standard procedure. The values employed were calculated from an average of at least 10 individual photometer readings.

oped and spontaneous recovery ensued. The potassium decrease in the serum of these 3 dogs was 53%, 37%, and 36% respectively. Table I shows a typical result on one of the dogs.

TABLE I

Specimen	mg.-%				Per cent decrease potassium	Remarks
	Magnesium	Calcium	Sodium	Potassium		
Initial	1.5	10.3	340	19.0		Normal
After 2 g. MgSO ₄	14.6	10.5	340	15.7	17	Breathing slightly abnormal. Legs showing some weakness.
After 4 g. MgSO ₄	24.5	10.9	339	14.3	25	Paralyzed. Breath coming in gasps. Rate 4/min.
15 min. later	16.7	11.6	330	13.2	31	Still paralyzed. Breathing abnormal. Rate increased to 24/min.
30 min. later	14.0	10.0	330	12.9	32	Can hold head up. Breathing still quite abnormal.
1 hr. later	11.4	9.9	335	12.1	36	Breathing still abnormal and dog has developed a characteristic cough.
6 hr. later	3.3	9.3	340	18.5		Practically normal except for an occasional cough and less activity.

ACKNOWLEDGMENTS

I am profoundly grateful to Dr. Ivan Brown for his encouragement and for placing at my disposal his Beckman spectrophotometer, to Miss Alberta Thiele for the calcium determinations, and to Mr. Thomas Lasater for other technical aid.

CONCLUSIONS

Thus, in six consecutive experiments on dogs paralyzed by magnesium sulfate administered parenterally, there has occurred, coincident with the sharp rise in serum magnesium, a decided drop in the serum potassium level of from 34 to 53% with no appreciable change in the serum levels of sodium and calcium. This suggests that some of the unexpected fatalities in man following intravenous magnesium therapy may not have been due to the elevated serum magnesium alone, but possibly to secondary effects on some of the other serum electrolytes. Conversely, some of the beneficial effects from intravenous magnesium therapy in syndromes with abnormally high serum potassium levels may have followed a reduction in this electrolyte.

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Erratum

In Table II, p. 472, of the article by Eiger and Greenstein, Vol. 19, No. 3, the value for the wavelength in the second line should be 2400, instead of 1400.

Book Reviews

Practical Methods in Biochemistry. By FREDERICK C. KOCH, Frank P. Hixon Distinguished Service Professor Emeritus of Biochemistry, and Associate Professor MARTIN E. HANKE, University of Chicago, 5th ed., rev. The Williams and Wilkins Co., Baltimore, 1948. ix + 419 pp., 20 figs. 13 × 23 cm. \$3.00.

As stated in the first edition by the late Professor Koch, "This laboratory manual is intended to present for medical students the more important qualitative and quantitative chemical aspects of cell constituents, of cell activities and of the composition of blood, secretions and excretions." The book has undergone a considerable amount of change since I reviewed the third edition in 1942 for the *J. Chem. Education*.¹ According to the authors, the present edition has been expanded in respect to material on manometric methods, microbiological methods for vitamins and amino acids, and also on colorimetric and fluorometric methods.

The book contains 16 chapters as well as an appendix and an index. The chapters are divided into Part I, The Chemistry of Cell Constituents; Part II, The Chemistry of the Digestive Tract; and Part III, Blood and Urine.

Ch. 5 covers hydrogen ion activity and pH. This chapter is very well written and should be valuable to every chemist.

Mett's method for the quantitative determination of peptic activity is given in Ch. 7. The reviewer considers this method to be obsolete. In this chapter it is stated on p. 133 that rennin acts in neutral solution, whereas rennin has practically no activity at pH 7.0 and its activity increases with increase of acidity.

In Ch. 8, dealing with intestinal digestion, it is stated on p. 137, ". . . others consider trypsin to be a definite compound of trypsinogen plus enterokinase." This is, of course, wrong and the activation of trypsinogen should be brought up to date as described by Northrop in his book "Crystalline Enzymes."

In Ch. 10, which deals with blood and hemoglobin, it is stated on p. 151 that methemoglobin is a union of oxygen and hemoglobin from which the gas cannot be pumped out, notwithstanding the fact that it is stated at the bottom of the next page that ferrieyanide decomposes oxyhemoglobin to form methemoglobin and gaseous oxygen.

On p. 153, reduced hematin is stated to be the same as hemochromogen and this mistake is repeated on p. 154.

Many methods are given for the quantitative analysis of blood in Ch. 11, and of urine in Ch. 12. This allows a considerable degree of choice, since there always has been and always will be a great difference of opinion among biological chemists as to which methods are the best.

The book is more valuable because colorimetric and fluorometric methods for vitamins are described in Ch. 14, microbiological methods in Ch. 15, and chemical tests for hormones in Ch. 16.

¹ Sumner, J. B., *J. Chem. Education* **19**, No. 3 (1942).

The appendix consists of 61 pp. of general directions for laboratory work. This is an excellent idea and certain to be of great aid to the laboratory worker. Also included in this chapter are logarithm tables and directions for making up reagents.

The index includes both subjects and authors, which is quite permissible for a relatively small book.

In summary, the reviewer believes that the fifth edition of *Practical Methods in Biochemistry* is an excellent book for both the beginner and the research worker. It contains a wealth of valuable information not likely to be easily obtained elsewhere.

JAMES B. SUMNER, Ithaca, N. Y.

Studies on Carbohydrate and Fat Metabolism, with Especial Reference to the Pigeon. By OSCAR RIDDLE and associates, Department of Genetics, Carnegie Institution of Washington. Carnegie Institution of Washington Publication 569, Washington, D. C., 1947. 128 pp. Price \$1.85 in paper cover; \$2.25 in cloth binding.

Many an innocent young biochemist may be enticed by this attractive title to settle into his chair for a pleasant evening's reading about a laboratory animal long used for research. Such a biochemist, however, will soon find himself in a 50 ring circus of endocrinology. The only biochemistry will be hundreds of analyses of blood sugar, liver-lipides, and glycogen.

The work really concerns numerous endocrine extracts, primarily those of the pituitary. By analyses of effects of extracts upon variations in blood sugar, the authors hoped to determine whether some of their extracts contained one or more individual components. Their hopes were seldom realized.

The blood sugar of the young Carneau pigeons used in these studies varied normally from 186 to 256 mg.-%.

Fractions of pituitary extract from ammonium sulfate were tested and found to have different effects upon heat production, lipogenesis and ketosis. Almost any single fraction would provide an average biochemist with a life time of work carrying out more exacting chemistry from the crude beginnings of these biologists.

Alkaline extracts were also prepared and tested from the posterior lobes of beef pituitary glands. These seemed to have slight effect on the carbohydrate and fat metabolism.

Numerous preparations were tested for their effect upon the blood lipid level. A few of these were pituitary hormone, corticotrophin, prolactin, estrone, and various other preparations.

The dizzy biochemist emerges from this "circus" with an increased appreciation of the intricacies of mechanisms in control of the lipid and sugar metabolism. However, most of the numerous researches were exploratory and permit few definite conclusions.

CLIVE M. McCAY, Ithaca, N. Y.

Advances in Enzymology, and Related Subjects in Biochemistry, Vol. VIII. Ed. F. F. NORD. Interscience Publishers, Inc., 1948. 538 pp. Price \$8.00.

The latest addition to this valuable series contains 10 articles which cover a wide range of subjects. The papers which deal with classical enzymology are definitely in the minority in this volume, while those which are concerned with subjects which pro-

vide the raw material for the future study of enzymatic mechanisms make up the bulk of the contributions. The articles will be discussed in the order of their appearance.

Monné contributes one of the longest articles, and, perhaps, one of the least satisfying. The subject, functioning of the cytoplasm, is certainly one of the most basic in biochemistry, yet it is reviewed in a rather parochial manner. In this instance, the cell is treated as being almost synonymous with the unfertilized sea urchin egg, as though the tissues of higher organisms are not made up of cells. Such subjects as contractility and conductivity are discussed as characteristic of all living cells. Yet vertebrate muscle and nerve, which perform these functions *par excellence* and whose study has yielded most of our information, are mentioned only in passing. Similarly, permeability is discussed in less than 4 pages; the treatment is so general as to be almost valueless. The most interesting section of this review is the portion devoted to cytoplasmic structures and their functions.

The exceedingly complex subject of complement is reviewed by Heidelberger and Mayer. It is good to see the many bewildering studies in this field reduced to a quantitative basis. These reviewers are hopeful that the hypothesis of an enzymatic mechanism of hemolysis may soon be susceptible of experimental attack.

The preparation and properties of the dehydropeptides and the dehydropeptidases are discussed by Greenstein, one of the most active investigators of this subject. This interesting field has been neglected until very recently. Considerable attention is now being devoted to these enzymes, and to the possible metabolic origin and function of the dehydropeptides.

Chaikoff and Entenmann discuss the present status of the anti-fatty liver factor of the pancreas. They believe that this syndrome, fatty livers in the depancreatized dog, is due to a failure of the animal to assimilate methionine from ingested intact protein. Hydrolyzed protein or methionine prevents the development of the disorder.

The problem of alkaloid biogenesis is carefully and critically reviewed by Dawson. It is apparent that our knowledge of intermediary plant metabolism is extremely meager. None of the enzymatic steps involved in the synthesis of these compounds has yet been identified. This able presentation of the problems should stimulate more interest in this field. It is unfortunate that there has been so little encouragement of fundamental investigations in plant biochemistry. An important practical side of plant chemistry, the microbiological degradation of cellulose, is discussed by Nord and Vitucci.

Good reviews which complement one another are presented by Kleinzeller on synthesis of lipides, by Breusch on fatty acid catabolism, and by Bergström and Holman on lipoxidase and the autoxidation of unsaturated fatty acids. In these 3 articles, one finds the entire field nicely summarized. It is an excellent feature of this volume to have these related topics treated side by side. It might be desirable to adopt a definite policy in succeeding volumes of dealing with different facets of a broad subject in the same volume.

Zeller writes on the enzymes of snake venoms and their biological significance. The efforts to identify the mechanism of action of the toxic principles of the venoms have received great impetus from the study of the enzymes in these fluids. Among the enzymes which have been found in the venoms are choline esterases, hyaluronidases, L-amino acid oxidases, lecithinases, and proteases.

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